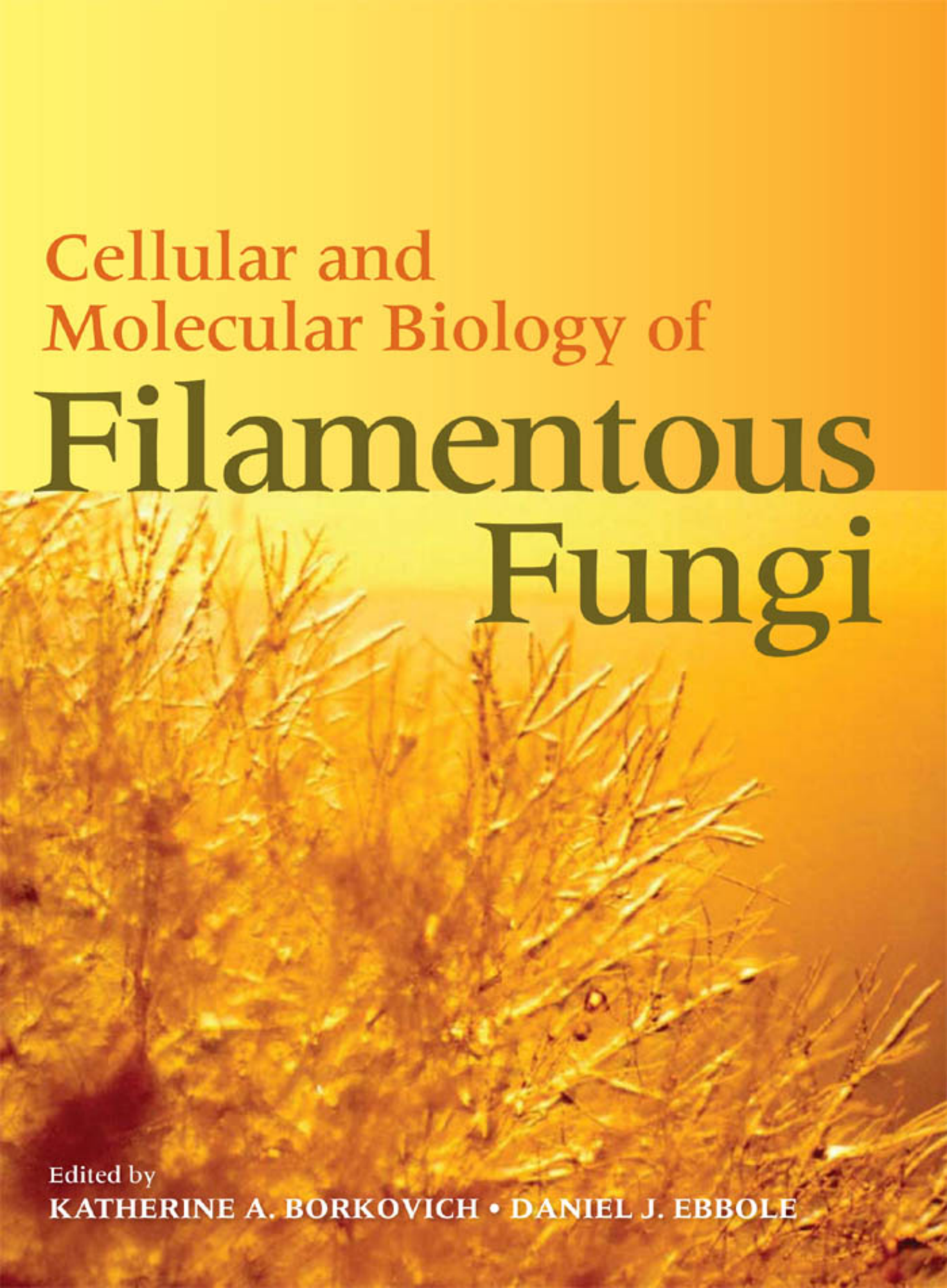
Cellular and Molecular Biology of Filamentous Fungi



Edited by
KATHERINE A. BORKOVICH • DANIEL J. EBBOLE

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Cover image: *Neurospora crassa* on Vogel's minimal medium plate at 30°C. Photo by F. Douglas Ivey.

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Preface

Fungal cellular and molecular biology has entered an unprecedented phase of rapid growth and change. The availability of complete genome sequences, coupled with the advent of new genetic and molecular tools, has made possible analyses that were deemed unthinkable just a few years ago. In the current environment, it is advantageous to have access to books that synthesize and rationalize the new information gleaned from genomics with the accumulated literature from years of research in the field.

Our goal was to organize a book that will be a major work on the filamentous fungi, serving as the first point of reference for this important group of organisms. We envisioned producing a handbook for professional researchers and graduate students in the field, as well as a textbook for graduate and undergraduate courses. Although model organisms are covered in detail, we strove to represent the diversity of the fungal kingdom. The chapters in this book cover a variety of dimorphic and "obligate" filamentous fungal species, illustrating lifestyles adapted to diverse ecological niches. Chapters also provide comparisons to work with the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, as appropriate. Every effort has been made to incorporate new data from genomics-based approaches and to provide well-grounded evaluation of the literature.

We are indebted to the many authors who made this book possible. All took time out from already overextended schedules to write chapters that are comprehensive and capture the essence of their field. In particular, we would like to acknowledge the efforts of Dr. Rowland Davis, Professor Emeritus at UC Irvine. Rowland suffered a severe stroke, yet emerged from a coma and extended time on a ventilator (during which time he could not speak) to ask about his reference list and permission to republish forms for figures! Rowland, you are an inspiration to the rest of us.

We recognize Dr. Michelle Momany for her efforts during the early stages of organizing the book. She was instrumental in developing the original outline and contacting prospective authors. New administrative duties as department head prohibited her from continuing with the project.

We thank ASM Press for having the foresight to solicit a book on filamentous fungi. We are indebted to Senior Editor Gregory Payne for first discussing the idea for this book with one of us (Borkovich) and then for his patience and advice as we solicited and read chapters and worked with the authors to create the best possible book. We thank Senior Production Editor Ellie Tupper, whose attention to detail and editorial standards are unsurpassed and are reflected in the final product.

Lastly, we would like to acknowledge those scientists (and mentors) who have passed away in the last few years and whose work laid the foundation for the information described in many of the chapters of this book. Among these are John Fincham, Norman Giles, Edward Garber, David Perkins, Dorothy Newmeyer, David Stadler, and Robert Metzenberg. Their enthusiasm for science and the study of filamentous fungi lives on in their scientific "descendants."

KATHERINE A. BORKOVICH AND
DANIEL J. EBBOLE, *EDITORS*

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INTRODUCTION TO THE FILAMENTOUS FUNGI

I

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Cellular and Molecular Biology of Filamentous Fungi
Edited by K. A. Borkovich and D. J. Ebbole

1

History and Importance to Human Affairs

JOAN W. BENNETT, ROWLAND DAVIS, AND CARLENE RAPER

The fungi have figured in folklore, myth, and art since ancient times. In 185 BCE, Nicander described a fungus as "the evil ferment of the earth." Centuries later, European herbalists speculated on the origin of such *earthy excrescences*, calling them bastard plants derived by spontaneous generation (Ramsbottom, 1953). Some believed fungi emanated from thunder. According to Ramsbottom, Caesalpinus described these organisms in 1583 as follows:

> Some plants have no seeds; these are the most imperfect, and spring from decaying substance; and they therefore have to feed themselves to grow. . . They are a sort of intermediate existence between plants and inanimate nature.

In the 18th century Linnaeus classified fungi as bona fide organisms belonging to a subgroup within the plant kingdom. Until a few decades ago, fungi were grouped with "cryptogams" (plants without flowers or seeds). Academics who studied them generally resided within Departments of Botany or Colleges of Agriculture. Now known to resemble animals more than plants, true fungi have been split off from other heterotrophic cryptogams such as the slime molds and Oomycota and are placed in a kingdom of their own (Barr, 1992; Baldauf and Palmer, 1993; Alexopoulos et al., 1996). Scientifically, they are studied primarily for their impact on human affairs and for what they can tell us about fundamental biological principles. In popular culture, many amateurs collect macrofungi for their food, hallucinogenic, tinder, or dye properties. A number of delightful books for popular reading are available, such as *The Romance of the Fungus World* (Rolfe and Rolfe, 1925); *Magical Mushrooms, Mischievous Molds* (Hudler, 1998); *Slayers, Saviors, Servants, and Sex. An Expose of the Kingdom Fungi* (Moore, 2001); and *Mr. Bloomfield's Orchard* (Money, 2002).

Fungi possess all the basic attributes of eukaryotes, including membrane-bound nuclei, chromatin, mitochondria, vacuoles, and cytoskeleton. Like plants, they grow in a polar manner, and walls, chemically different from those of plants, bind their cells. Physiologically, however, they resemble animals more than plants. They also encompass unique characteristics. Perhaps their single most distinct feature relates to nutritional strategy. Fungi, as heterotrophs, literally live in both their food and waste. Lacking chlorophyll, they generally use a variety of carbon and nitrogen sources to make virtually all the vitamins and amino acids necessary for life. They inhabit a broad spectrum of environmental niches spanning a wide range of food sources, temperature, and moisture. They travel primarily through spore dispersal. While vegetative fungal cells are essentially aquatic, their differentiated spores, whether vegetative or sexual, can survive dry, harsh environmental conditions over extensive periods of time. Fungal spores are found in the air at great heights all over the world.

Because of their nutritional versatility, fungi perform many essential roles in global ecosystems. As carbon recyclers, they figure importantly in biomass degradation and in helping plants forage nutrients through the formation of mycorrhizae (Isaac, 1992). On the other hand, many forms feed off crop plants. Fungal blasts, blights, and mildews have been known since the beginning of agriculture. Chestnut blight, Dutch elm disease, potato light blight, wheat rust, corn rust, rice blight, and coffee rust are all caused by various species of fungi. In fact, the vast majority of plant pathogens are fungi (Large, 1940; Money, 2007).

Several species attack animals as well. It is not widely appreciated that Agostino Bassi, an Italian scientist working well before Pasteur and Koch, proposed a microbial theory of disease based on his studies of a fungal disease of silkworms called muscardine. Following earlier work on sheep breeding and cheese production under such luminaries as Spallanzani and Volta at the University of Padua, Bassi spent several years studying muscardine. By 1835 he had identified the causative agent as a fungus similar to *Botrytis* that could transmit the disease through infection of its powdery spore mass from diseased insects to healthy ones. Ten years later, Bassi extended his contagion theory to human diseases such as cholera, gangrene, plague, rabies, typhus, and smallpox. He also published on the importance of chemical disinfection and the use of heat for sterilization.

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Pasteur later cited Bassi in several of his writings and is said to have kept a copy of Bassi's portrait in his office (Major, 1944; Porter, 1973). Unfortunately, most introductory microbiology books do not mention Bassi's groundbreaking contribution to germ theory.

While few fungi are obligate animal pathogens, some facultative species cause severe illness in humans, particularly in those who are immunocompromised. Among these are *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, and *Histoplasma capsulatum*. Up-to-date research on these animal pathogens as well as several plant pathogens is presented in this volume on filamentous fungi.

In addition to their ability to cause disease, many fungi damage materials useful to mankind by invading stored foods, fabrics, lumber, cellulose, and even plaster and cement. A few species have been identified as possibly related to "sick building syndrome." Others contaminate postharvest crops and produce toxic substances. Yet in truth, most fungi are harmless, even helpful to humankind. Sometimes their degradative powers can be harnessed for bioremediation (Bennett et al., 2001). They are also important to a number of industries in the processing of food, paper, alcohol, drugs, and dyes; in the production of enzymes, organic acids, and antibiotics; and, of course, in the harvesting and cultivation of edible mushrooms (Tkacz and Lange, 2004; An, 2005).

The fungal kingdom provides a diversity of approachable models for studying an array of biological phenomena. A general capacity for making most of the essential vitamins and amino acids permits culturing of many species on defined media under controlled conditions in the laboratory. Short, predominantly haploid life cycles with propagation dependent on either asexual or sexual spore disposal facilitate genetic studies. A unique capacity for heterokaryosis (the mitotic reproduction of different types of haploid nuclei within a common cytoplasm) eases analyses of genetic complementation. With such advantages, the fungi provide a wealth of model systems for basic research on eukaryotic gene regulation, mitochondrial inheritance, senescence, cell cycle control, metabolism, signal sensing, circadian rhythms, and population genetics (Maheshwari, 2005).

Notable among these model systems is the ascomycete *Neurospora crassa*. George Beadle and Edward Tatum used this fungus to investigate how genes control metabolism, the complex series of enzymatic reactions required for making life-sustaining molecules (reviewed by G. W. Beadle [1948]). Beadle teamed first with Boris Ephrussi on experiments with the genetically tractable fruit fly *Drosophila melanogaster* but soon found that organism too complex to complete the goal in mind. Learning from earlier studies by B. O. Dodge and C. C. Lindegren about the unique advantages of *N. crassa* (then known as *Neurospora sitophila*, a contaminant of bakeries and sugar cane processing), Beadle, a corn geneticist, engaged Tatum, a biochemist, to employ this fungus instead. Using a simple defined medium to isolate a variety of nutritionally deficient mutants (auxotrophs), Beadle and Tatum capitalized on *N. crassa*'s haploid nature and its capacity for heterokaryosis and meiotic recombination to promulgate their famous "one gene one enzyme" hypothesis. Why not choose humans for such a study? While metabolic pathways can be studied in humans, the latter are not ideal organisms for examining gene function. As George Beadle put it (in a gender-biased phrasing of the time), "His life cycle is too long, his offspring are too few, his choice of a mate is not often based on a desire to contribute to the knowledge of heredity, and it is inconvenient to subject him to a complete chemical analysis." (Beadle, 1948). Beadle and Tatum received the Nobel Prize for their pioneering study of *Neurospora*. It opened a whole new field of biochemical genetics that extended to other organisms and reveals striking commonality in metabolic pathways throughout the biological world.

For the most part, Beadle and Tatum confirmed their hypothesis that single genes encode single enzymes, but later studies revealed exceptions. Using the heterokaryon complementation test to determine whether two mutations reside in one or more genes, Fincham and colleagues discovered that two particular mutants defective for the production of NADP-glutamate dehydrogenase in *N. crassa* complemented one another yet the mutations responsible resided within a single gene (Fincham and Pateman, 1957). They showed that this enzyme is a homomultimer, constituted of multiple polypeptides, all encoded by the *gdh* gene. Such exceptions seem to be confined to multimeric proteins. Studies with *Neurospora* and another ascomycete, *Aspergillus nidulans*, demonstrated that genes of a given metabolic pathway are usually scattered, not clustered within operons as in bacteria—an early insight on differences in gene arrangement between eukaryotes and prokaryotes. Subsequent studies on gene regulation of metabolic pathways revealed regulatory genes that initiate a cascade of gene activity. For example, when nitrate is substituted for ammonia in the medium of *N. crassa*, the *nit-3* gene encoding nitrate reductase is induced. This transcriptional response requires the activity of two genes, first *nit-2*, and then *nit-4*, which is induced by the presence of nitrate. When nitrogen sufficiency is achieved, *nit-3* transcription is turned off indirectly by the *nmr* gene product which binds with glutamine to block the action of the *nit-2* gene product (Marzluf, 1997). Many such Rube Goldberg schemes have been worked out first in the fungi, including yeast (*Saccharomyces cerevisiae*). Comparable examples of gene regulation have been shown to exist in more complex eukaryotes.

The advantages of working with *Neurospora*, *Aspergillus*, and other filamentous fungi are clearly evident in formal genetic studies, and there are several excellent textbooks that provide reviews of classical fungal genetics (Esser and Kuenen, 1967; Fincham et al., 1979; Bos, 1996). Early work by B. O. Dodge and Carl and Gertrude Lindegren during the 1920s and 1930s had established *N. crassa* as an organism "with absolutely orthodox genetics" (Ryan and Olive, 1961; Davis, 2000). The asci of *N. crassa* contain octads of ascospores. Meiosis yields a linear tetrad in which the products of the first meiotic division remain in either the upper or lower halves of the tetrad; the products of the second division remain adjacent in each half, and sister spores remain adjacent. Robin Holliday, using the technique of tetrad analysis in *N. crassa*, developed an important model of DNA recombination. The rare aberrant ratios of 6:2 or 5:7 rather than the 4:4 ratio expected for segregation of two alleles of a single gene were explained as events of gene conversion. Holliday observed that gene conversion correlated positively with recombination and suggested that conversion took place not at single mutational sites but in conversion tracts (segments of chromosome). He posited that such conversion was a by-product of meiotic recombination, resulting in loss of information in one chromosome and its repair through use of the homologous chromosome as template during synapsis. Holliday thus proposed an innovative model of DNA strand exchange known as the Holliday junction (Holliday, 1964).

The foundations of mitochondrial genetics and mitochondrial function originated with studies involving the

yeast *S. cerevisiae* and the filamentous *N. crassa*. Both fungi have served significantly in our understanding of vacuolar function and the role that this organelle plays in nitrogen and phosphate storage and amino acid metabolism (Mitchell and Mitchell, 1952; Davis, 2000).

Since the earlier classical studies, fungal research has progressed rapidly with the advent of molecular biology. It is now possible to extract, manipulate, and amplify genes for reintroduction into most model fungi and thus determine cause and effect from genotype to phenotype. While such manipulations can apply to human cell cultures, they are not as easily manageable in the living, breathing human.

Application of the molecular toolbox to model fungal systems has advanced our understanding of a host of biological principles described in this volume. For example, genetic control of the eukaryotic cell cycle, first studied in depth in the yeast *S. cerevisiae*, was also probed in *A. nidulans* by Ron Morris and associates. They identified genes responsible for the mechanisms of nuclear migration and cellular growth that suggested comparable mechanisms for nuclear migration in developing neurons of the mammalian brain (Morris, 1975, 2000).

Nevertheless, *N. crassa* remains the dominant filamentous fungal model, in part because of the strong scientific community involved in its use (Davis, 2007). For example, finite mechanisms of gene silencing with parallels in other organisms have been elucidated in *N. crassa*. The first discovered is the premeiotic repeat induced point mutation (known as "RIP") phenomenon. It was discovered by inserting stretches of DNA identical to resident DNA into the genome and noting that both the introduced and resident copies become heavily mutated by methylation, thus destroying function (Singer and Selker, 1995). Other schemes of gene silencing, also discovered in *N. crassa*, involve RNA. Quelling, which is related to RNA interference in animals, occurs posttranscriptionally in DNA-transformed cells through the combined action of RNA-dependent RNA polymerase and other (dicer) components (Catalanotto et al., 2004; Fulci and Macino, 2007).

Details on the stages of meiosis have come from research with *Coprinus cinereus* (recently renamed *Coprinopsis cinerea*). Mimi Zolan and Pat Pukkila have taken advantage of a unique attribute of this basidiomycete. Its process of meiosis can be synchronized by an appropriate regime of light versus dark (Pukkila et al., 1985). Light sensing figures prominently in the discovery of the underlying mechanisms of circadian rhythms. Jay Dunlap, Jennifer Loros, and associates have led the way in a detailed molecular analysis of the numerous genes and proteins responsible for *N. crassa*'s physiological accommodation to the light-dark cycle that affects all organisms. Their work laid the groundwork for extended studies in other organisms such as the fruit fly and humans, in which numerous commonalities have been revealed (Bell-Pedersen et al., 1996; Dunlap, 1999).

Signaling and sensing are central to mate recognition among the fungi. An astonishing range of sexual expressions exists throughout the kingdom. Following Blakeslee's 1904 discovery of a sexual cycle with two mating types in the Mucorales, a host of studies defined a variety of sexual mechanisms in other species. Notable among these were the independent works of Kniep (1920) and Bensaude (1918), who first described an incompatibility system with multiple sexes in the mushroom-bearing basidiomycetes *Schizophyllum commune* and *Coprinus lagopus* (synonym *Coprinopsis cinerea*). John and Carlene Raper, Lorna Casselton, and their associates extended these studies leading to the molecular investigations included in this volume.

The fungi express seven different patterns of sexuality. Raper bemusedly speculated that such multifarious lifestyles suggest that Mother Nature used the fungi as a testing ground for all possible ways to accomplish sex. This wealth of sexual mechanisms provides fortuitous material for exploring the genetic and molecular controls of the sexual process. John Raper wrote a book called *Genetics of Sexuality in the Higher Basidiomycetes* over 40 years ago (1966), in which he aptly noted, "Perhaps the most important development in the evolution of the fungi was the emergence of the complex and interrelated features of incompatibility, heterokaryosis and the incorporation of the dikaryon as an integral phase of the sexual cycle." Joe Heitman, James Kronstad, John Taylor, and Lorna Casselton edited the recently published book, *Sex in Fungi* (2007), documenting the enormous progress made in this field since Raper's book. In light of more recent discoveries, the underlying mechanisms of sexual diversity, noted by Raper decades ago, involve molecules of ancient origin that figure prominently in development and sensing throughout the biological world.

We now know that the elaboration of multiple mating types (thousands in the Higher [Homo] Basidiomycetes) evolved from two genetic complexes regulating the two mating types in the Ascomycetes, Hemiascomycetes, and Hemibasidiomycetes. One set of genes encodes transcriptionally regulating homeodomain proteins comparable to other homeodomain proteins that regulate development and differentiation in many animals including worms, flies, and humans (Casselton and Kues, 2007). The other set of genes encode pheromones and pheromone receptors similar to molecules that regulate odor, taste, and sight in flies and mammals (Fowler and Vaillancourt, 2007). Bipolar mating type systems predominate in the Ascomycota, while the Homobasidiomycetes have numerous versions of these important regulator genes. Interestingly, the pheromone and receptor genes of the homobasidiomycete *S. commune* can be expressed in the hemiascomycete *S. cerevisiae* by using the yeast's native processing enzymes, thus indicating important connections between distantly related species (Fowler et al., 1999). Research with the model species *S. commune* and *Coprinopsis cinerea* provides unique opportunities for understanding how so many different versions of these genes function at the molecular level to detect compatible mates.

Not all fungi engage in sex. Among the currently defined 100,000 species, well over 17,000 have no known sexual state (Hawksworth et al., 1983; Hawksworth and Rossman, 1997). These "asexual" fungi are variously called anamorphic, mitosporic, "the fungi imperfecti," or Deuteromycetes. Before the use of nucleic acid characters in taxonomy, they posed serious challenges to classification (Reynolds and Taylor, 1993). They include many microfungi important in agriculture, industry, and medicine. *Aspergillus niger* is one such species. Pontecorvo and colleagues (1953) discovered a rare process of genetic recombination in this fungus, in which haploid nuclei occasionally fuse, form somatic diploids, and then proceed through a series of haploidizations. They called it parasexuality. Using *A. nidulans*, a species with a sexual phase that taxonomists and GenBank call *Emericella nidulans*, the Pontecorvo group demonstrated the value of parasexual analysis for mapping genes onto chromosomes, a method used for decades in mammalian cell genetics (Pontecorvo, 1956). This process also proved useful for genetic mapping in other asexual species capable of heterokaryosis, such as members of the antibiotic-producing penicillia (MacDonald, 1983) and aflatoxin-producing

species of *Aspergillus* (Bennett and Papa, 1988). Recombinant DNA manipulations, gene cloning, expression libraries, transcriptional profiling, and the sequencing of entire genomes have been a boon to genetic analysis of all fungi, but particularly to those lacking a sexual cycle (Bennett and Lasure, 1985, 1991; Prade and Bohnert, 2003; Goldman and Osmani, 2008).

Genome projects create databases that greatly accelerate the process of gene discovery across the biological world (http://www.genomesonline.org/). Not surprisingly, the power of entire genomes has proven irresistible to the mycological community. The last 10 years have seen an explosive growth in the number of fungal species that have genome projects (http://www.genomesonline.org/gold.cgi?want=Eukaryotic+Ongoing+Genomes). Genome projects have been particularly useful for well-developed genetic systems such as *N. crassa* (Borkovich et al., 2004) but also for poorly characterized nonsexual species such as *A. fumigatus* (Nierman et al., 2005). However, in general, our ability to exploit these voluminous in silico data has lagged behind our capacity to acquire them, and this increasing gap has highlighted the importance of experimental manipulations in biology.

This volume is a testament to the continuing robustness of fungal systems as a source of insight into all of the life sciences.

Rowland Davis actively and enthusiastically organized the early stages of planning this chapter, but because he suffered a serious stroke on May 11, 2008, he was unable to participate in the writing. J.W.B. and C.A.R. thank him for his inspiration but take responsibility for any errors of content and style in this final version.

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2

Hyphal Structure

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Structure is a fundamental element of the physical world. It is so basic that we can often overlook its profound significance. Structure exists at all levels of organization, from atomic particles to the universe. There is a continuum of structured levels with a higher-level structure often composed of multiple copies of a lower-level structure. Concepts such as order, connection, morphology, recognition, organization, and function are all associated with the perception of structure. How structure originates, develops, matures, reproduces, evolves, reacts, and ultimately dies is the motivation underlying most approaches of inquiry and discovery in the sciences (Pullan and Bhadeshia, 2000).

The defining feature of filamentous fungi is the hypha. Hyphal growth provides the means for colonization of substrates, secretion of hydrolytic enzymes, nutrient assimilation, regulation of morphogenesis, and recognition of environmental signals. Hyphae also function as gametes in sexual reproduction in many fungi and differentiate into a variety of structures such as the paraphyses in ascomycete fruiting bodies; thick-walled, clampless skeletal and binding hyphae, cystidia, and setae in basidiomycete fruiting bodies; and infectious structures such as appressoria and haustoria.

Hyphal growth and differentiation are complex processes requiring the control of cell wall synthesis, polarized vesicle transport, exocytosis, endocytosis, turgor pressure, organelle positioning, and cytoplasmic migration and fusion. The result is a polarized system of growth giving rise to a tubular cell. The components that make up the cytoplasm of hyphae, and for that matter all cells, are assembled into a dynamic, interconnected, and structured unit that provides the foundation for growth and morphology. Indeed, all aspects of cellular behavior are supported by the underlying structure of the cytoplasm. The hyphal cytoplasm contains many of the organelles and subcellular inclusions found in other heterotrophic eukaryotic organisms. However, because of their mode of growth and diverse interactions with the ecosystem, hyphae also maintain a cytoplasmic structure and contain organelles and inclusions that are unique to the fungi. Some features show significant structural plasticity within the fungi themselves or are present only in certain fungal groups. Such features (e.g., Spitzenkörper, cytoskeletal order, septal structure, and nuclear division) are of particular importance in understanding aspects of hyphal behaviors and have been used as indicators of evolutionary relationships. Recently, a community level effort and a multigene approach have resulted in a stable phylogenetic classification of fungi to the level of order (Hibbett et al., 2007). As part of this effort, the Assembling the Fungal Tree of Life Structural and Biochemical Database (AFTOL, 2007) has been established as a developing repository of images and references to studies on the topic (Celio et al., 2006).

Much of our knowledge of hyphal structure has come from decades of intensive study of these cells by light microscopy (LM) and transmission electron microscopy (TEM). In the late 1950s and 1960s the work from several laboratories illustrated the utility of bioimaging methods in elucidating hyphal cytoplasmic structure. In 1957, Girbardt described cytoplasmic organization and behavior in living hyphae of *Trametes versicolor* (Basidiomycota) by using phase-contrast LM (Girbardt, 1957). This work was coupled to ultrastructural studies using TEM and three-dimensional reconstruction methods (Hawker, 1965; Bracker, 1967; Girbardt, 1969) providing an unprecedented view of hyphal structure. A few years later Grove and Bracker (1970) used LM and TEM to make a significant impact on understanding cytoplasmic order and emphasized its diversity among major fungal groups. These works and others paved the way

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for further structural studies aimed at understanding hyphal growth, morphology, and fungal taxonomy and evolution (Kahn and Kimbrough, 1982; Kumar et al., 2007). The use of TEM for structural studies was strengthened in the late 1970s with the application of cryo-methods for ultrastructural investigations (Howard and Aist, 1979). When successful, ultrafast freezing and sub-zero degree dehydration significantly improves the preservation of many cell types and their subcellular structures compared to traditional preparation protocols, such as aqueous chemical fixatives and room temperature dehydration (Chandler and Roberson, 2009). As more researchers took advantage of these improvements, knowledge of fungal cellular structure grew rapidly (e.g., Howard, 1981; Vargas et al., 1993). The most recent advancement in ultrastructural research has been the application of electron tomography for high-resolution and three-dimensional analysis of internal cellular structures (Müller et al., 2000; Hohmann-Marriott et al., 2006). By the mid-1980s, the advancement in ultrastructural studies was supported greatly by the development of new tools for LM and live-cell imaging. Using high-resolution cameras, computer technology, and fluorescently conjugated molecular probes, the study of many structural components in space and time became possible (Spellig et al., 1996; Suelmann et al., 1997; Freitag et al., 2004; Mouriño-Pérez et al., 2006; Uchida et al., 2008).

Equipped with a better knowledge of cellular structure, and interfacing this with the strength of molecular biology, significant advancements can be made towards understanding the biology of a cell and organism. In this chapter, structural features of hyphae from diverse taxonomic groups are reviewed. Also addressed are characteristics of cytoplasmic order and selected organelles and features that are important in studies of hyphal growth and fungal phylogeny.

HYPHAL ORGANIZATION

The constitutive delivery of secretory vesicles from their sites of origin to the point of growth is carried out in a hyphal cytoplasm that maintains a highly polarized order (see also chapter 19). Hyphae maintain this polarity in order to sustain three main processes: synthesis of the cell wall, growth of the plasma membrane, and secretion of materials required for nutrition. The establishment and maintenance of cytoplasmic polarity is not unique to the fungal hypha. In fact, it is a fundamental characteristic of cell biology as evidenced by asymmetric cell division, cell motility, and the establishment of cell surface features (e.g., microvilli and flagella). The most obvious examples of cytoplasmic polarity found in most hyphae are the maintenance of the Spitzenkörper (i.e., the apical body) (see "Spitzenkörper" below) located at the growing apex of the cell and the alignment of most organelles and structures along the axis of growth. An example of polarized cytoplasmic order is illustrated in a hypha of *Neurospora crassa* (Ascomycota) and *Sclerotium rolfsii* (Basidiomycota) (Fig. 1).

The hyphal tip of most fungi can be partitioned into several regions based on cytoplasmic order. However, depending on the particular fungal taxon this order can vary. The apical dome, or region I (McDaniel and Roberson, 2000), begins at the tip of the cell and typically extends back 2 to 3 μm. This limited area is structurally simple; however, it is the site of highly active processes that are fundamental to hyphal growth and morphogenesis (e.g., vesicle motility, exocytosis, cytoskeletal dynamics, and cell wall biosynthesis). Mitochondria, occasionally smooth endoplasmic reticulum (ER), and in the Ascomycota, Woronin bodies are found in region I. As mentioned above, the primary structure at the apex of growing hyphae of septate fungi (i.e., Ascomycota and Basidiomycota) is the Spitzenkörper (Fig. 1 to 3). A structural equivalent of the Spitzenkörper is not common or has not been observed in hyphae or hypha-like cells (e.g.,

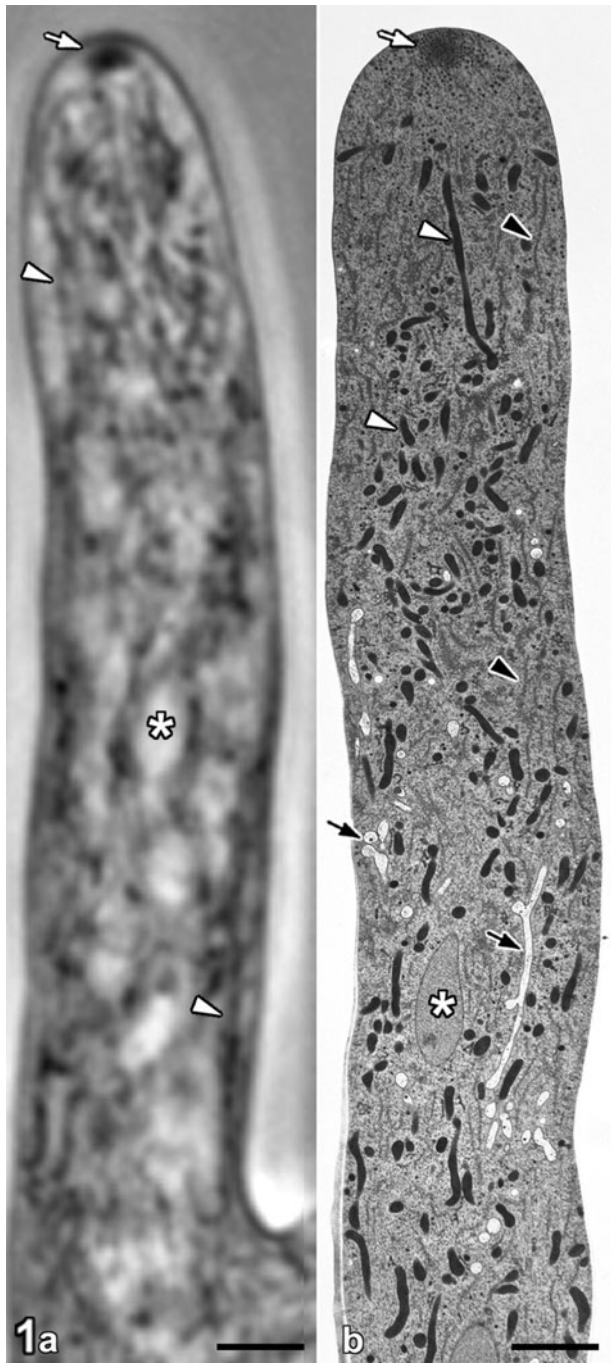

FIGURE 1 Cytoplasmic order of hyphal tip. (a) Phase-contrast light micrograph of *N. crassa* with Spitzenkörper (white arrow), mitochondria (arrowheads), and nucleus (asterisk). Scale bar, 4 μm. (b) Near-median TEM section through *S. rolfsii* cryofixed hypha illustrating Spitzenkörper (white arrow), mitochondria (white arrowheads), rough ER (black arrowheads), vacuoles (black arrows), and nucleus (asterisk). Scale bar, 2 μm. Reprinted from *Protoplasma* (Roberson and Fuller, 1988) with permission from the publisher.

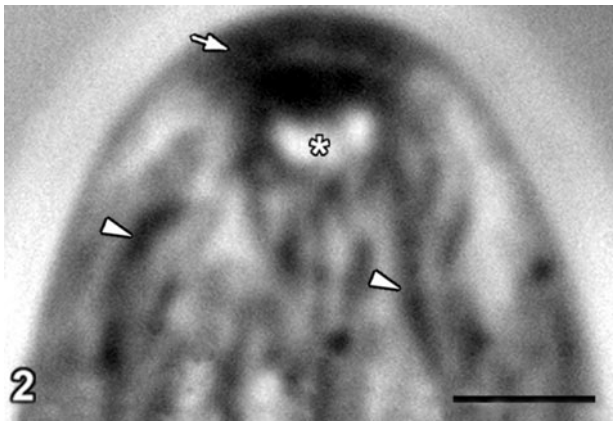

FIGURE 2 Phase-contrast light micrograph of *N. crassa* hyphal tip illustrating the phase-dark region (arrow) and the subtending phase-light core of Spitzenkörper. Mitochondria are indicated by arrowheads. Scale bar, 1.5 μm.

rhizoids) of the zygosporic fungi (e.g., Mucoromycotina) or the other relatively early diverging lineages. As a notable exception to this statement, Spitzenkörper have been identified in hyphae of *Allomyces macrogynus* (Blastocladiomycota) (Vargas et al., 1993), a zoosporic fungus that produces hyphae and rhizoids. In the fungi and fungus-like organisms (e.g., Oomycota) that lack Spitzenkörper, clustering of vesicles is seen near the apical plasma membrane. Although the arrangement of these vesicles appears to be less organized and complex than those of the Spitzenkörper of Ascomycota and Basidiomycota, there are characteristic patterns that are recognized. For example, in *Rhizopus* species and other members of the Mucorales (Mucoromycotina), a thin crescent-shaped band of closely packed vesicles is present just beneath the apical plasma membrane (Grove and Bracker, 1970). That Spitzenkörper are found only in the fungi, and primarily only in the mature hyphae of septate fungi, begs questions regarding its evolution and role in polarized growth.

Below the apical dome, the hyphal cytoplasm contains common eukaryotic inclusions and organelles including nuclei, cisternae and vacuoles, rough and smooth ER, mitochondria, cytoskeletal elements, vesicles, ribosomes, and Golgi equivalents. In the septate fungi, typically two subapical regions are recognized, regions II and III. Region II is the subapical zone just behind the apical dome, and in optimally growing hyphae, it can extend from 15 to 25 μm (Riquelme et al., 2002). In hyphae growing at less than optimal rates, the size of region II is reduced. Region III extends subapically behind region II until the first septum appears. The primary feature that differentiates region II from region III in both septate and aseptate fungi is the presence of nuclei, region II being devoid of nuclei.

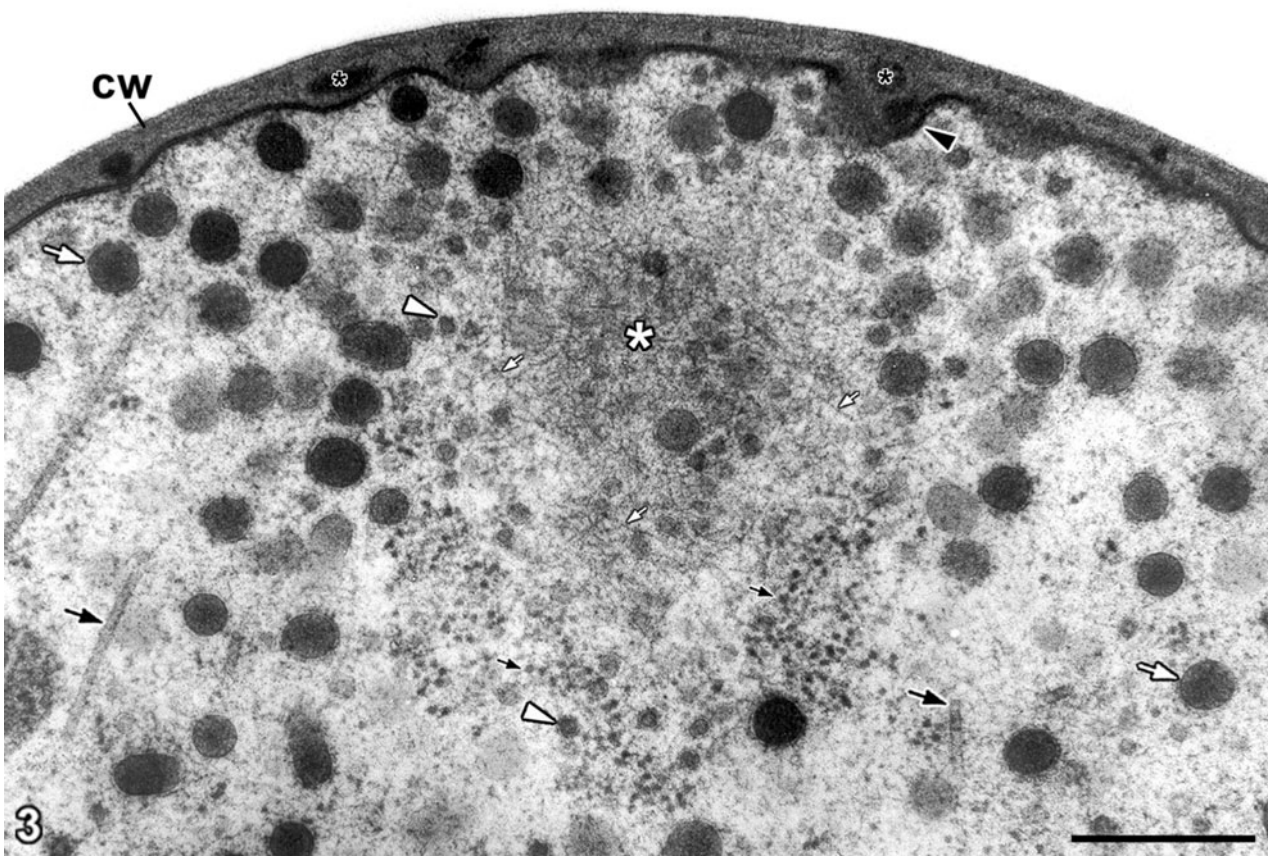

FIGURE 3 Near-median TEM section through hyphal tip of *Botrytis cinerea* (Ascomycota) illustrating structural components of the Spitzenkörper and surrounding cytoplasm. Shown are apical vesicles (white arrows); microvesicles (white arrowheads); central core (asterisk); MTs (black arrows); MFs (small white arrows); plasma membrane at sites of exocytosis (black arrowheads); ribosomes (small black arrows); cell wall (CW); cell wall inclusions (black asterisks). Scale bar, 200 nm.

Analogous to the hypha is the rhizoid, a filamentous structural feature of the thallus that occurs frequently among early-diverging zoosporic fungi. A rhizoid is a relatively short, thin somatic branch of a thallus, usually with determinate growth. It contains limited cytoplasm and lacks nuclei and Spitzenkörper. Rhizoidal elongation results from unipolar development of a zoospore cyst: the rhizoid develops as an extension of the zoospore cyst and ramifies over or within a substrate. Rhizoids are also found in certain members of zygospore-producing fungi, particularly parasites of invertebrate animals. The function of a rhizoid is similar to that of a hypha in that it accesses and transports nutrients to a developing reproductive body and anchors the organism to a substrate.

CELLULAR COMPONENTS: STRUCTURE, FUNCTION, AND ORGANIZATION

Spitzenkörper

The Spitzenkörper is a dense, spheroidal cluster of vesicles, cytoskeletal components, and signaling proteins found at the tips of most growing hyphae. It plays crucial roles in optimizing hyphal extension rates and in determining patterns of growth and morphogenesis by acting as a dynamic organizing center in the reception of secretory vesicles from subapical regions and orchestrating their delivery to the apical membrane (i.e., a "'vesicle supply center'") (Bartnicki-García et al.,1989; Riquelme et al.,1998). In the strict sense, the Spitzenkörper is a manifestation of the secretory pathway (see "Endomembrane System: Secretory and Endocytic Pathways," below). The Spitzenkörper is not bound by a membrane and can exhibit significant variability in size, shape, position, and behavior. These variations can occur between cells of the same species due to the natural dynamic pleomorphism of the Spitzenkörper and/or responses to endogenous and environmental signals that influence the numbers and types of vesicles that associate with it. The Spitzenkörper is an ephemeral structure, and its presence and absence are tightly correlated with rates of cell growth. It assembles as secondary and tertiary hyphal branches emerge (Riquelme and Bartnicki-García, 2004) and as germ tubes mature into hyphae (Araujo-Palomares et al., 2007).

In observations with phase-contrast microscopy of septate fungi, the Spitzenkörper typically appears as a dark body that partially or completely encloses a differentiated light central core (Fig. 1 and 2). Nine morphological patterns of Spitzenkörper organization have been described using phase-contrast microscopy (López-Franco and Bracker, 1996). Whether morphological patterns can be clearly articulated ultrastructurally and represent valid phylogenetic markers remains to be determined.

The most obvious component of the Spitzenkörper of the Basidiomycota and Ascomycota is a dense aggregation of secretory vesicles, the ultrastructural equivalent of the dark region seen with phase-contrast microscopy (Fig. 1 to 3) (Girbardt, 1969; Grove and Bracker, 1970; Howard, 1981). The Spitzenkörper central core contains substantially fewer vesicles and thus has a lower refractive index, giving rise to its phase-light appearance. Morphologically there are two categories of vesicles: the apical vesicles, also known as macrovesicles (70 to 100 nm in diameter), and smaller polyhedral microvesicles (30 to 40 nm in diameter). Although the arrangements of Spitzenkörper vesicles may appear to be disordered and random, spiral and linear patterns of vesicles have been observed. For example, Roberson and Fuller (1988) reported linear patterns of microvesicles associated with microfilaments (MFs), presumably filamentous actin. The Spitzenkörper is, in fact, enriched with actin MFs that are most concentrated within the core (Fig. 3) (Bourett and Howard, 1991). Microtubules (MTs) are also found associated with the Spitzenkörper (Fig. 3). A thin bed of ribosomes (Fig. 3) subtends the Spitzenkörper and appears to be a consistent feature in the Spitzenkörper of the Ascomycota.

Sites of exocytosis at the hyphal apex are rarely visualized when hyphae are prepared for electron microscopy using cryofixation methods, although exocytotic profiles are abundant in hyphae fixed with standard chemical methods (Howard and Aist, 1979). It is believed this is due to the high rate at which heat must be withdrawn from cells in order to achieve freezing without resolvable ice crystal damage, a process that requires fixing the cells within milliseconds (Chandler and Roberson, 2009). Figure 3 is a rare image of a cryofixed cell showing numerous sites of exocytosis at the apical plasma membrane. Spherical to ovoid inclusions within the cell wall are possibly the contents of vesicles just after exocytosis. This image suggests that vesicle fusion with the plasma membrane may occur in synchronized bursts of exocytosis.

Although, as mentioned above, Spitzenkörper are present in all growing hyphae of the Ascomycota and Basidiomycota, they are not common in other fungi. Thus far, Spitzenkörper have been confirmed in only one other fungal phylum, the Blastocladiomycota in *Allomyces* (Fig. 4 and 5) (Vargas et al., 1993). The *Allomyces* Spitzenkörper is unique both structurally and functionally. The most distinctive aspect of the *Allomyces* Spitzenkörper is its large (~3-μm) phase-light core (Fig. 4). Also, since there is no evidence for apical vesicles in *Allomyces*, the phase-dark region present in Fig. 4 is created primarily by closely associated mitochondria (Fig. 5). The ultrastructural equivalent of the phase-light area contains a granular-to-fibrous matrix with an irregular boundary in which MFs, microvesicles, and MTs are present (Fig. 5). The *Allomyces* Spitzenkörper is also set apart from those of the septate fungi in that it contains γ-tubulin and functions as an MT organizing center (MTOC) (Roberson and Vargas, 1994; McDaniel and Roberson, 1998). The interest in the diversity of structure and function of the *Allomyces* Spitzenkörper is amplified by the fact that members of the

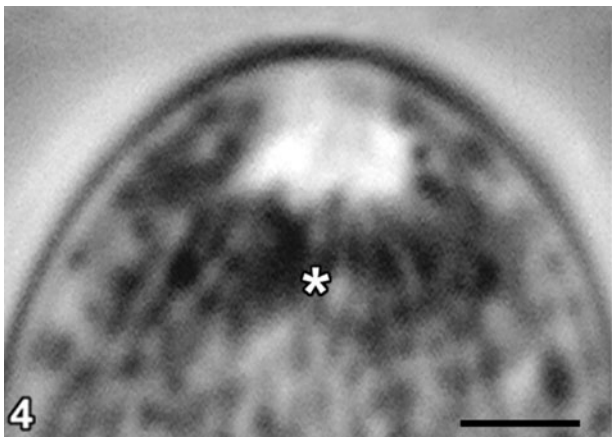

FIGURE 4 Phase-contrast light micrograph of *A. macrogynus* hyphal tip illustrating phase-light core of Spitzenkörper subtended by mitochondria (asterisk). Scale bar, 4 μm. Reprinted from *Fungal Genetics and Biology* (McDaniel and Roberson, 2000) with permission from the publisher.

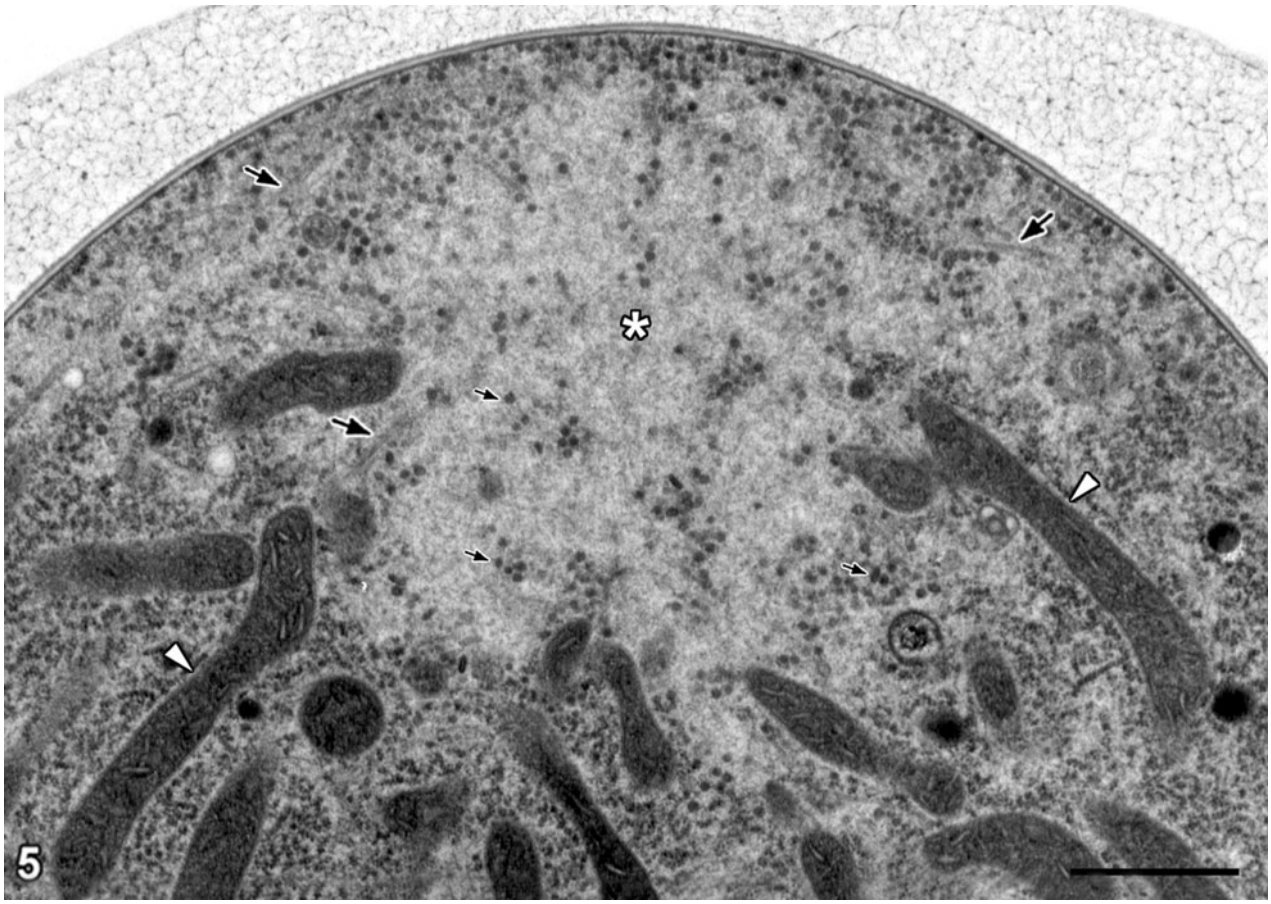

FIGURE 5 Near-median TEM section through hyphal tip of *A. macrogynus* illustrating structural components of the Spitzenkörper and surrounding cytoplasm: central core (asterisk); microvesicles (small arrows); MTs (arrows); and mitochondria (arrowheads). Scale bar, 1.7 μm. Reprinted from *Fungal Genetics and Biology* (McDaniel and Roberson, 2000) with permission from the publisher.

Blastocladiomycota are zoosporic fungi. Unlike nonzoosporic fungi, they possess centrosomes rather than spindle pole bodies (SPBs) as their nuclear-associated MTOC. Indeed, this group of fungi may prove to be pivotal in understanding the evolution of the Spitzenkörper as fungi became adapted to a terrestrial lifestyle. Further investigations of zoosporic and aseptate hyphal fungi may result in additional evidence of diverse Spitzenkörper.

Nuclei

Interphase nuclei in filamentous fungi are structurally similar to those in other eukaryotic organisms. They have a nuclear envelope perforated by numerous nuclear pore complexes enclosing a ribosome-free nucleoplasm packed with chromatin of varying densities and containing one or two nucleoli (Fig. 6). An MTOC is associated with the cytosolic surface of the nuclear envelope, and in nonzoosporic fungi this is a SPB (Fig. 6 and 7a through d). In the zoosporic fungi the nuclear MTOC is a centrosome, and, as in animal cells, it is composed of a pair of barrel-shaped centrioles positioned at a 90° angle relative to each other and embedded in a pericentriolar matrix. *Basidiobolus* is a fungus of great interest, as it has a modified centrosomal MTOC yet has lost the ability to produce flagella (McKerracher and Heath, 1985). A diversity of SPB forms occur among the zygosporic fungi, which reflects their diverse evolutionary origins (Celio et al., 2006). In many Basidiomycota the SPB is modified into a globular form (Fig. 7c and d) from the plaque or disk-shaped structure characteristic of the Ascomycota, a form also present in some Basidiomycota (Pucciniomycotina) (Fig. 7a and b), reflecting shared ancestry. The shape of nuclei varies from approximately spherical in older, more quiescent cellular regions to elongate or pyriform in those regions where rapid cell growth or cytoplasmic motility is occurring. Nuclei are extremely malleable and able to pass through very small openings, such as septal pores, enabling their movement from one cell into another. Fungal nuclei range in size from 1 to 3 μm in diameter; except those of *Basidiobolus*, which can be up to 25 μm in length.

The number of nuclei per hyphal compartment varies depending on the fungal group. In the dikaryotic (n+n) Basidiomycota there are typically two nuclei per compartment after plasmogamy, and these are maintained in close proximity to each other (Fig. 6). Movement into new hyphal compartments may be achieved via the formation of a clamp connection during mitosis and cytokinesis. Free nuclear migration from one compartment to another is prevented by the complex septal dolipore apparatus (see "Septal Structure," below), unless there has been enzymatic breakdown of septa after plasmogamy. In the haploid Ascomycota, each hyphal

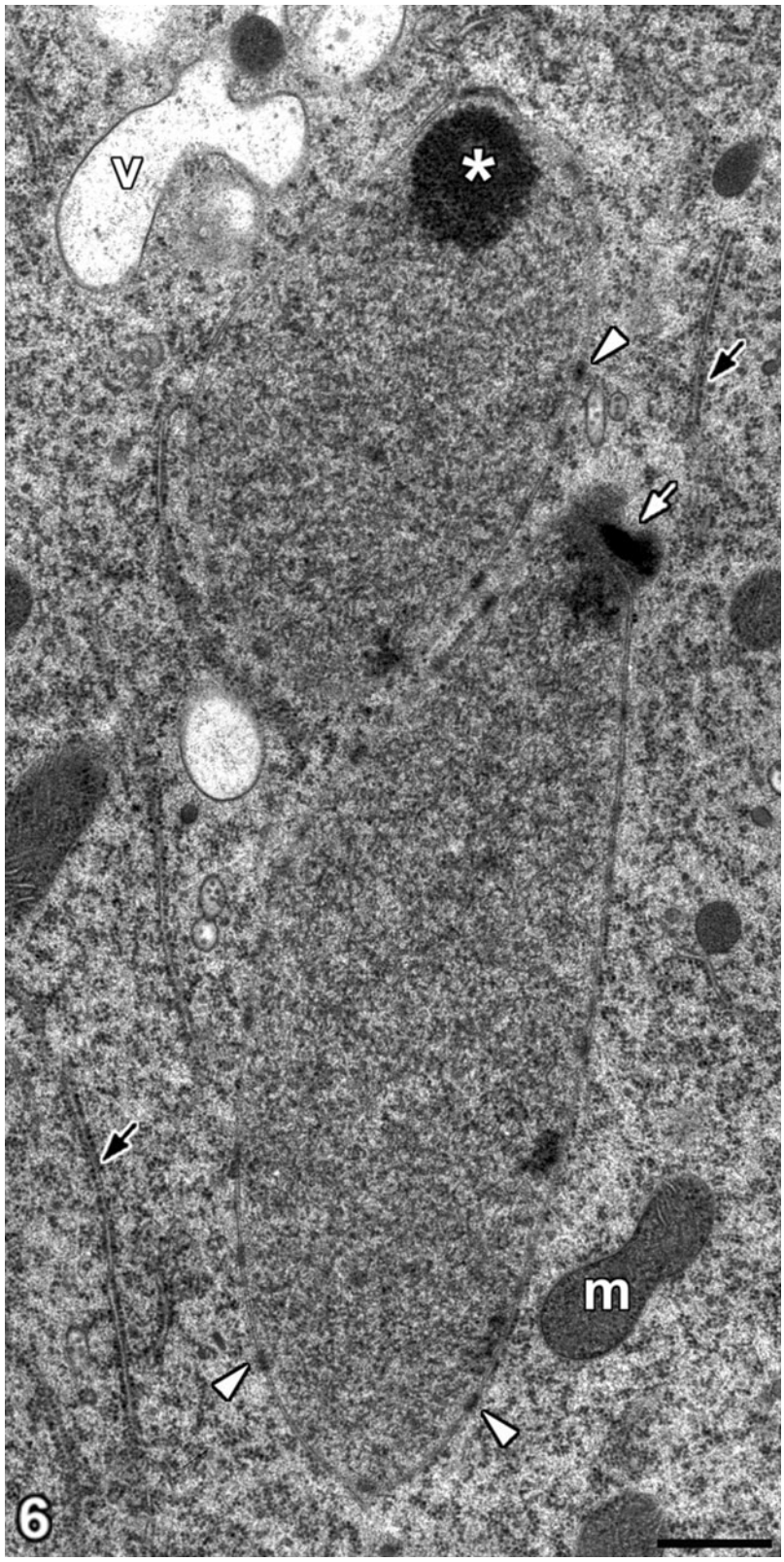

FIGURE 6 TEM of nuclei of *S. rolfsii* illustrating prominent nucleolus (asterisk), nuclear pore complexes (white arrowheads), SPB (white arrow), mitochondrion (m), rough ER (black arrows), and vacuole (V). Scale bar, 350 nm.

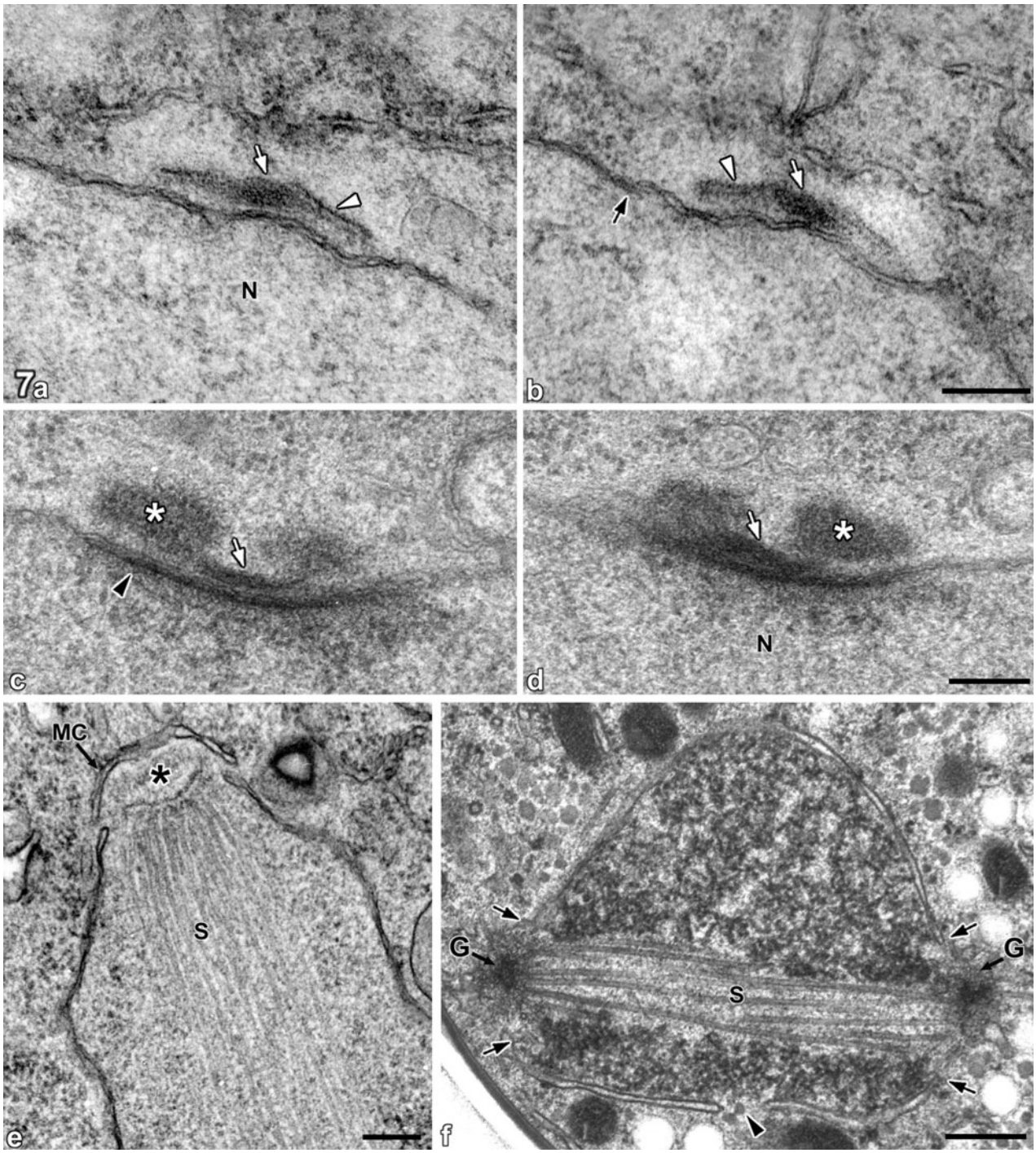

FIGURE 7 TEM of SPB and mitosis. (a and b) Sections 3 and 5 of a complete series of 6 through a longitudinally sectioned late interphase SPB of *Helicobasidium mompa* showing the disk form (arrowheads) of the SPB characteristic of Ascomycota and Pucciniomycotina, Basidiomycota. Shown are the middle piece of SPB (white arrows), nuclear envelope (black arrow), and the nucleus (N). Scale bar, 0.2 μm. Reprinted from the *Canadian Journal of Botany* (Bourett and McLaughlin, 1986) with permission of the publisher. (c and d) Sections 3 and 4 of a complete series of 8 through a late prophase SPB of *Auricularia auricula-judae* (Agaricomycotina, Basidiomycota) showing the two globular elements (asterisks) and middle piece (white arrows) surrounded by a ribosome-free zone; an intranuclear element (black arrowhead) consisting of two layers lies within the nucleus (N) adjacent to the nuclear envelope. The intranuclear element is characteristic of many members of Ascomycota and Basidiomycota. Scale bar, 0.2 μm. Reprinted from the *Canadian Journal of Botany* (Lu and McLaughlin, 1995) with permission of the publisher. (e) The metaphase SPB of *H. mompa* is a disk (asterisk) set in a pore in the nuclear envelope surrounded by a membranous cap (MC). S, spindle. Scale bar, 0.2 μm. Reprinted from the *Canadian Journal of Botany* (Bourett and McLaughlin, 1986) with permission of the publisher. (f) The metaphase SPB of *Auriscalpium vulgare* (Agaricomycotina, Basidiomycota) is a globular element (G) with a dense inclusion lying in a large polar fenestra (delimited by arrows) and a mostly continuous nuclear envelope with a few gaps (arrowhead). Scale bar, 0.5 μm. Reprinted from *Mycologia* (Celio et al., 2007) with permission of the publisher.

compartment can contain one to many nuclei; however, a large number of nuclei may be found in the apical compartment before septum formation. The nuclei are more or less free to move from one compartment to the other through a simple septal pore, unless it is plugged (see "Woronin Bodies," below). In the fungi that lack septa or have few septa, large numbers of nuclei are distributed throughout the cytoplasm. These fungi are considered coenocytic.

The history of the study of mitosis in filamentous fungi was reviewed by Aist and Morris (1999) (see also chapter 6). They described the considerable understanding of the structures involved in mitosis by 19th and early 20th century researchers who extrapolated from the results of light-microscopic studies of large meiotic nuclei to the smaller nuclei characteristic of mitotic divisions. A period of confusion in the understanding of fungal mitosis, which characterized the 1950s and 1960s, was followed by a series of studies using newly available ultrastructural methods to show that fungal mitosis was comparable to that in other eukaryotes, albeit with some features characteristic of fungi (Aist and Morris, 1999). These features include absence of a metaphase plate, a usually intact nuclear envelope during division, asynchronous separation of chromosomes in anaphase A, elongation of a narrow central spindle in anaphase B, and synchronized mitosis in haploid filamentous fungi (Fig. 7e and f).

The number of mitotic studies within a single phylum of fungi is limited, and generalizations based on these limited studies may prove to be unfounded (Celio et al., 2006). The best-studied phylum is the Basidiomycota, which shows considerable variation in the organization of the nuclear envelope during metaphase when a number of taxa are sampled. The structures may vary from an SPB loosely inserted in a pore in the nuclear envelope with a membranous cap at metaphase surrounding the SPB on its cytoplasmic face (Fig. 7e) without (Bourett and McLaughlin, 1986) or with perinuclear ER, to loss of the nuclear envelope with the retention of the membranous cap (Swann et al., 1999), to an intranuclear SPB with a more or less closed nuclear envelope, to an SPB lacking a membranous cap and tightly or loosely inserted in a polar fenestra (Fig. 7f) (Celio et al., 2007). In the Ascomycota the metaphase nuclear envelope is either intact, as in most basal phyla with the exception of the Chytridiomycota, or with a polar fenestra (Celio et al., 2006). The functional significance of these variations in membrane organization is unknown. However, astral MTs fail to form while membranous caps are present, and this suggests that they may regulate polar movements.

A benefit from studies that provide a more complete understanding of the variations in mitosis is that they can yield new insights into the mechanisms of nuclear division and challenge our present concepts. For example, it is usual to find that during mitosis the SPBs are approximately parallel to each other at each end of the spindle, but in *Kriegeria eriophori* (Basidiomycota) the poles may be perpendicular to each other during division with unknown implications for the mitotic mechanism (Swann et al., 1999).

Mitochondria

Like that of nuclei, the structure of fungal mitochondria is similar to that of other eukaryotic organisms (see chapter 14). They are bound by a double-unit membrane with the internal membrane having a much increased surface area that is manifested in elaborate invaginations (i.e., cristae) that extend into the mitochondrial matrix. Fungal mitochondria differ from other eukaryotes in that their shape is more elongate, they are larger organelles, and their plate-like cristae are arranged as lamellae that are parallel to the organelle's long axis. Mitochondria occur throughout the hyphal tip cytoplasm and can be particularly dense in numbers near the apex, where large amounts of ATP are required to sustain growth (Fig. 1, 2, 4, 5, and 8). They are typically aligned along the long axis of the hypha and are often seen in close proximity to MTs, rough ER, and Golgi equivalents. Mitochondria move mostly at bulk flow rates; however, they are capable of rapid saltatory motility in filamentous fungi via actions of MTs (Fuchs and Westermann, 2005) or actin MFs (Suelmann and Fischer, 2000) and their associated motors (Fig. 8).

Endomembrane System: Secretory and Endocytic Pathways

The endomembrane system is a cytoplasmic association of membranes that divide the cell into functional and structural organelles and compartments that together provide a means for the synthesis, breakdown, and transport of

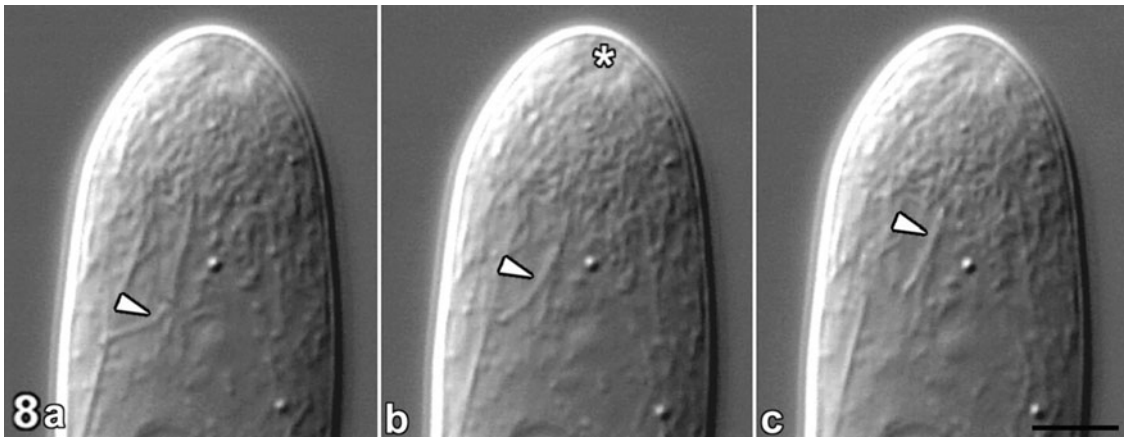

FIGURE 8 Differential interference contrast LM of *A. macrogynus* illustrating saltatory motility of a mitochondrion (arrowhead) presented over a 1.5-s period. The Spitzenkörper is indicated by an asterisk. Bar, 5 μm. Reprinted from *Fungal Genetics and Biology* (McDaniel and Roberson, 2000) with permission from the publisher.

materials out of, into, and within the cell. The components of the endomembrane system are connected by the intracellular movements of materials via vesicles or, less commonly, physical connections (e.g., nuclear envelope and rough ER). Below, we discuss briefly elements of the secretory and endocytic pathways. For a recent review on the protein constituents of each organelle please see Bourett et al. (2007).

Secretory Pathway

The outward secretory pathway starts with the synthesis of proteins at the rough ER and from there through the Golgi apparatus to the cell surface. The ER is thought of as a unified interconnected membranous network with distinct functional domains and morphologies that play essential roles in membrane and protein biogenesis. It is present in all regions of the hyphal tip cell. The network generally exhibits a gradient distribution with increasing abundance towards the hyphal apex (Maruyama and Kitamoto, 2007). Extensive profiles of rough ER are present in hyphal subapical regions. These are easily recognized ultrastructurally when viewed in cross section as thin sheets of ribosome-decorated membrane cisternae oriented along the growing axis of the hypha (Fig. 1b and 9a). Smooth ER is less common, although it can be found in region I in close association with the Spitzenkörper and in association with dolipore septa of the Basidiomycota (see "Septal Structure," below). This organelle is involved in the synthesis of lipids, further processing of proteins, carbohydrate metabolism, and detoxification of drugs and poisons. Increased amounts of smooth ER occur in hyphae grown under stress conditions and in the presence of certain growth inhibitors (for an example, see Roberson and Fuller, 1990).

The Golgi apparatus is involved in additional modifications of proteins delivered from the rough ER and in their packaging into transport vesicles. In most eukaryotic cells this organelle is composed of a group of flattened cisternae, or dictyosomes, that are layered one on top of the other in a manner similar to a stack of pita bread with each cisterna having distinct roles in protein modification. In fungi the structure of the Golgi apparatus is unique and appears as inflated tubular and often fenestrated cisternae that vary in shape from cup-like to planar bodies (Fig. 9a through e). Because of the structurally simplified nature of the fungal Golgi apparatus, Morré and others (1971) referred to individual organelles as Golgi equivalents. However, they have physiological functions typical of stacked Golgi cisternae of other organisms (Sewall et al., 1989). Recently, Hubbard and Kaminskyj (2008) reported the localization of a Golgi marker (CopA) tagged with green fluorescent protein (GFP) in *Aspergillus nidulans* (Ascomycota). They showed that Golgi equivalents are more abundant at hyphal tips than in subapical regions. Vesicles, mitochondria, and MTs are often observed in close association with Golgi equivalents (Fig. 9b through e).

Vesicles are small spherical bodies bound by a single-unit membrane with a cargo of proteins and glycoproteins. Vesicles are found throughout the hyphal cytoplasm. They are most highly concentrated in the apex of growing hyphae in the area identified as the Spitzenkörper by LM. Clouds of vesicles are also seen in small aggregations near the plasma membrane in region I of the hyphal tip and are referred to as satellite Spitzenkörper (López-Franco et al., 1995). Satellite Spitzenkörper form de novo and combine with the primary Spitzenkörper, and they may be involved in producing pulsed hyphal growth (López-Franco et al., 1994). As is the case for all eukaryotic cells, fungal vesicles provide an invaluable link in the packaging and delivery of goods to designated targets. As would be expected, vesicles are often associated with cytoskeletal elements (for examples, see Roberson and Fuller, 1988; Riquelme et al., 2002; and Zekert and Fischer, 2009).

There are several classes of fungal vesicles; some are found throughout the Fungi while others are found in specific taxa. The apical vesicles and microvesicles have been described previously (see "Spitzenkörper," above) and are both likely secretory vesicles, although little is known regarding their composition and distinct roles in tip growth. Bartnicki-García and coworkers (1978) isolated a population of vesicles termed chitosomes from fungal hyphae and showed that they possessed chitin synthase activity. Chitosomes are similar in appearance to microvesicles, but without confirming immunoelectron microscopy their relationship has remained unclear. However, recent localization of GFP-labeled chitin synthases to the Spitzenkörper core of *N. crassa* (Riquelme et al., 2007) represents a significant step in addressing this question. Microvesicles are sometimes coated with a dense fibrous network referred to as filasomes (Fig. 9f) (Howard, 1981). The fibrous coat of filasomes contains actin (Bourett and Howard, 1991; Roberson, 1992) and other actin-binding proteins and likely represents, in part, the cortical actin patches seen with epifluorescence imaging (see "MTs," below). Filasomes are most abundant in the cortex of the apical hyphal regions and may be the manifestation of non-clathrin-facilitated endocytosis. Studies of fungal genomes (Read and Kalkman, 2003) and live-cell imaging experiments using endocytic marker dyes (for an example, see Peñalva, 2005) support the view that endocytosis is common in filamentous fungi.

Endocytic Pathway

The inward endocytic pathway starts at the plasma membrane and is responsible for lipid and protein recycling and for uptake and breakdown of extracellular materials through an endosomal and lysosomal system. Components of the endocytic compartments in filamentous fungi have been difficult to identify due perhaps to a reduced and less complex endocytic system relative to mammalian cells (Bourett et al., 2007). Recent studies using the membrane-selective fluorescent vital dye FM4-64 to visualize the endocytic pathway have broadened our understanding of the structure and dynamics of the endocytic compartments in filamentous fungi (Fischer-Parton et al., 2000; Ashford and Allaway, 2007). Multivesicular bodies (MVBs) are common in fungi and other eukaryotic organisms. In mammalian cells these organelles may be involved in the transfer of materials from early endosomes to lysosomes (i.e., mature endosomes/vacuoles in fungi) (Piper and Katzmann, 2007). They are recognized with TEM as membrane-enclosed bodies (150 to 350 nm in diameter) containing smaller vesicle-like particles and are often associated with MTs (Fig. 9g). At the LM level, these organelles are likely the small spherical bodies exhibiting MT-dependent bidirectional saltatory motion (McDaniel and Roberson, 2000; Riquelme et al., 2002). They probably are also components of the endosomal structures visualized in a number of fungal species after FM-464 staining and GFP tagging of early endosome components (for examples, see Fischer-Parton et al., 2000; Peñalva, 2005; and Abenza et al., 2009).

Fungal vacuoles are motile, complex organelles with versatile functions and morphologies (Ashford and Allaway, 2007) (see chapter 15). Vacuoles are analogous to mammalian lysosomes, playing a primary role in the breakdown of materials. In the fungi they also function as storage compartments for biosynthetic precursors, calcium ions, and

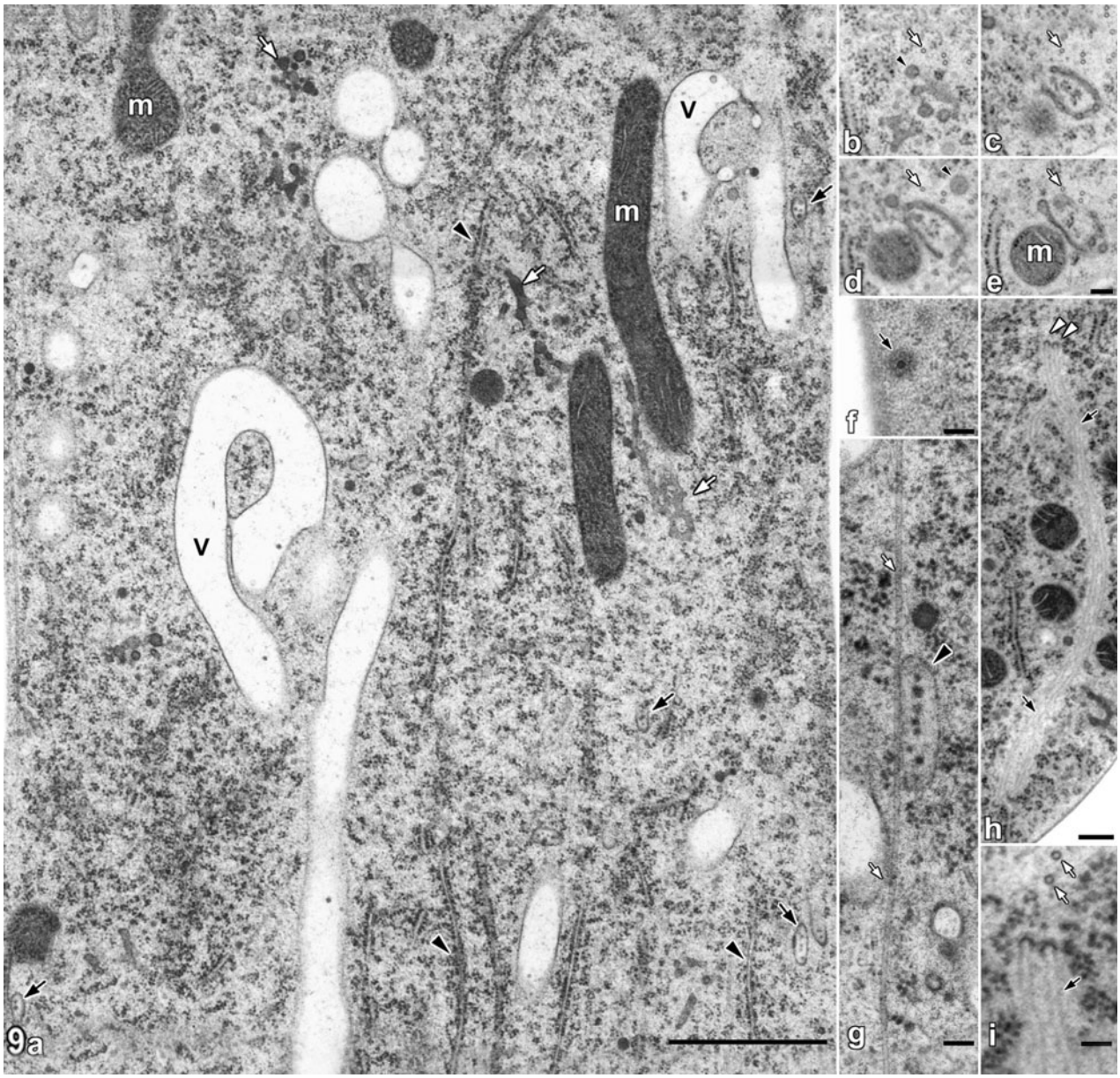

FIGURE 9 Elements of the endomembrane system and organelles viewed with TEM. (a) *S. rolfsii* hyphal region III showing vacuoles (V), inflated cisternae of Golgi apparatus (white arrows), rough ER (arrowheads), MVBs (black arrows), and mitochondria (M). Bar, 1.0 μm. Reprinted from *Protoplasma* (Roberson and Fuller, 2000) with permission from the publisher. (b through e) Serial cross sections through Golgi equivalent of *N. crassa* showing apical vesicle (arrowheads), MT (arrows), and a mitochondrion (M). Bar, 0.1 μm. (f) Filasome (arrow) in cortex of *N. crassa* hypha. Bar, 0.12 μm. (g) MVB (arrowhead) juxtaposed to MT (arrow) in *S. rolfsii*. Bar, 0.1 μm. From Roberson and Fuller (1988), reproduced with permission from Springer. (h) Flattened vacuolar cisternae (arrows). White arrowheads indicate coated surfaces of vacuoles. Hypha is cut in cross section. Scale bar, 0.2 μm. (i) High magnification of panel h showing coated surfaces of vacuole (black arrow) and cross section of two closely associated MTs (white arrows). Scale bar, 75 nm.

phosphates; in pH and osmotic regulation; and in ion homeostasis and cytoplasmic detoxification (Veses et al., 2008). Furthermore, they are involved in membrane recycling, long-distance nutrient transport through the fungal body, and regulation of hyphal extension. The distribution and morphology of vacuoles vary among the fungal taxa and also from cell to cell within individuals, depending on physiological conditions and cellular function. In many growing hyphal tip cells the vacuole is viewed as a dynamic network of small tubular-to-flattened cisternae that are continuously or transiently linked with larger spherical-to-elongate cisternae that group in clusters or are solitary (for examples, see Roberson and Fuller, 1988; and Ashford and Allaway, 2007) (Fig. 9a, h, and i). In some cases, the hypha can

possess a cytoplasm that is dominated almost entirely by a single vacuole that maintains a small "slug" of cytosolic contents near the growing tip. MTs are commonly in close association with vacuoles (Fig. 9i). Observations with TEM have identified electron-dense fibrillar material coating the cytosolic surfaces of elaborate cisternae identified as vacuoles (Fig. 9h and i). Vacuolar content varies depending on cellular maturation and health. During optimal growth the luminal content ranges from being electron transparent to containing a fibrous/granular matrix. Small spherical inclusions and sometimes crystalline inclusions are also present. Under conditions of environmental stress or exposure to growth inhibitors, vacuoles increase in number and size and their lumen becomes more electron dense and filled with inclusions (Roberson and Fuller, 1990).

Cytoskeleton

Cells regulate their morphology, organize and support cytoplasmic components, interact with the environment, and control their movements and intracellular transport. These functions are fulfilled through the coordinated actions of the cytoskeleton (see chapter 17). The fungal cytoskeleton is composed primarily of two structural elements, the MTs and actin MFs, which extend throughout the cytoplasm, making connections with themselves and other cellular elements to form a continuous interconnected cytoplasmic system. Each cytoskeletal element has discrete mechanical and dynamic characteristics and performs specific and shared duties in the cell. Cytoskeletal function and behavior are direct results of the inherent characteristics of their proteins as well as the actions of hundreds of associated proteins that interact with the cytoskeleton and cytoplasmic components in highly regulated and precise ways. In many eukaryotes there is a third component of the cytoskeleton, intermediate filaments. However, their abundance and role(s) in creating a self-stabilizing structural framework in the fungal cytoplasm is apparently much reduced due to the contributions of the fungal cell wall and turgor pressure, and they will not be addressed here.

MTs

MTs are tubular structures built from subunits of α- and β-tubulin heterodimers. These heterodimers assemble end to end, forming 13 parallel protofilaments that bundle together to build the wall of the MT. MTs have an outer diameter of ~24 nm and a variable length. The lumen of the MT has a diameter of ~14 nm and is routinely described as "empty." However, there is clear evidence that dense particles and fibrous materials reside within the core of the MT, which may represent MT-binding proteins that regulate its assembly and disassembly (Garvalov et al., 2006).

Each αβ-tubulin dimer has a structural polarity that is manifested throughout the entire MT polymer. One end of the MT has β-tubulin exposed and is referred to as the plus end, while the α-tubulin end is the minus end. The plus and minus designations refer to the rate at which αβ-tubulin dimers add to the MT. In vitro studies show that in a solution of pure tubulin, dimers assemble at both ends of the MT but add faster to the plus end. In the cell, MT nucleation and organization are regulated by the MTOC (e.g., SPB and centrosome) through the interactions of the MT's minus end with another member of the tubulin family, γ-tubulin (Oakley and Oakley, 1989). γ-Tubulin monomers combine with other proteins to form hundreds of ring-shaped aggregates (i.e., the γ-tubulin ring complex) that are embedded within the matrix of the MTOC. Thus, a single γ-tubulin ring complex serves as a nucleation site for a single MT that is oriented with its minus end attached to the MTOC and its plus end extending into and interacting with the cytoplasm.

MTs occupy the cortical and central cytoplasmic hyphal regions (Fig. 10), with most studies indicating their abundance in the cell cortex. In the subapical hyphal regions MTs are primarily oriented parallel to the growing axis of the cell, often exhibiting a distinct helical curvature with variable pitches that outline nuclei and mitochondria. MTs have a tendency to intertwine with one another to form a loosely braided meshwork in the central cytoplasm. This arrangement is most obvious in large mature hyphae (Mouriño-Pérez et al., 2006) (Fig. 10) and less so in smaller hyphae and germ tubes. Oblique or transverse MT orientations are observed during branch formation and in association with the mitotic spindle. Cytoplasmic MTs are mostly solitary, although bundles of two to four sometimes occur (Fig. 9b through e and 10) and are likely bound together by Tau-like associated proteins and/or molecular motors, although this remains equivocal (Fischer, 2007). MT bundles are transient structures providing increased cytoplasmic support and the potential for generating contractile forces when MTs are arranged with opposite polarities (e.g., mitotic spindles). As mentioned above, MTs are often in close proximity to membrane-associated structures (Fig. 3 and 9b through e and g) and, occasionally, are juxtaposed to actin MFs (Vargas et al., 1993).

Dynamic instability is the primary behavior of MTs in eukaryotic cells. It is characterized by the rapid interconversion between assembly and disassembly of the MT plus end and provides a means for MTs to reassemble into different structural organizations during cell cycle, growth, and development. In hyphal regions I and II of septate hyphae, dynamic instability is visualized in GFP-tubulin strains (Uchida et al., 2008) as the anterograde growth of MTs, many of which enter the apical dome and interact with the Spitzenkörper and apical plasma membrane with their plus ends. After a brief pause, MTs undergo a catastrophic disassembly and shrink until rescued, and the cycle repeats. Fragmentation of MTs and their subsequent anterograde and retrograde movements have been reported in the hyphal cortex of *N. crassa* (Uchida et al., 2008). These actions suggest the presence of MT-severing proteins (e.g., katanin) and treadmilling or active MT transport within this fungus.

LM and TEM (Fig. 3 and 10) (Hohmann-Marriott et al., 2006; Freitag et al., 2004; Mouriño-Pérez et al., 2006) imaging reveals that MTs tend to converge within the apical dome. The apical convergence of MTs is most pronounced in hyphae of *A. macrogynus* (Blastocladiomycota) because, as mentioned above, the Spitzenkörper serves as an MTOC in this fungus (Roberson and Vargas, 1994; McDaniel and Roberson, 1998). The idea that the Spitzenkörper might be an MTOC in all fungi is a very provocative one, but repeated attempts to localize γ-tubulin to the Spitzenkörper of septate fungi have failed (R. W. Roberson and R. R. Mouriño-Pérez, unpublished results). Furthermore, results from live-cell studies of GFP-tubulin fungal strains provided no indication that MTs are nucleated at the Spitzenkörper (Uchida et al., 2008), which would be expected from an MTOC. Although this role has not been established for other Spitzenkörper, there are reports of MT nucleation from apical sites in germ tubes of the rust fungus *Uromyces phaseoli* (Basidiomycota) (Hoch and Staples, 1985) and during lateral branch formation in *N. crassa* (Mouriño-Pérez et al., 2006).

Actin MFs

Actin MFs are composed of subunits of identical globular actin monomers that assemble into two protofilaments,

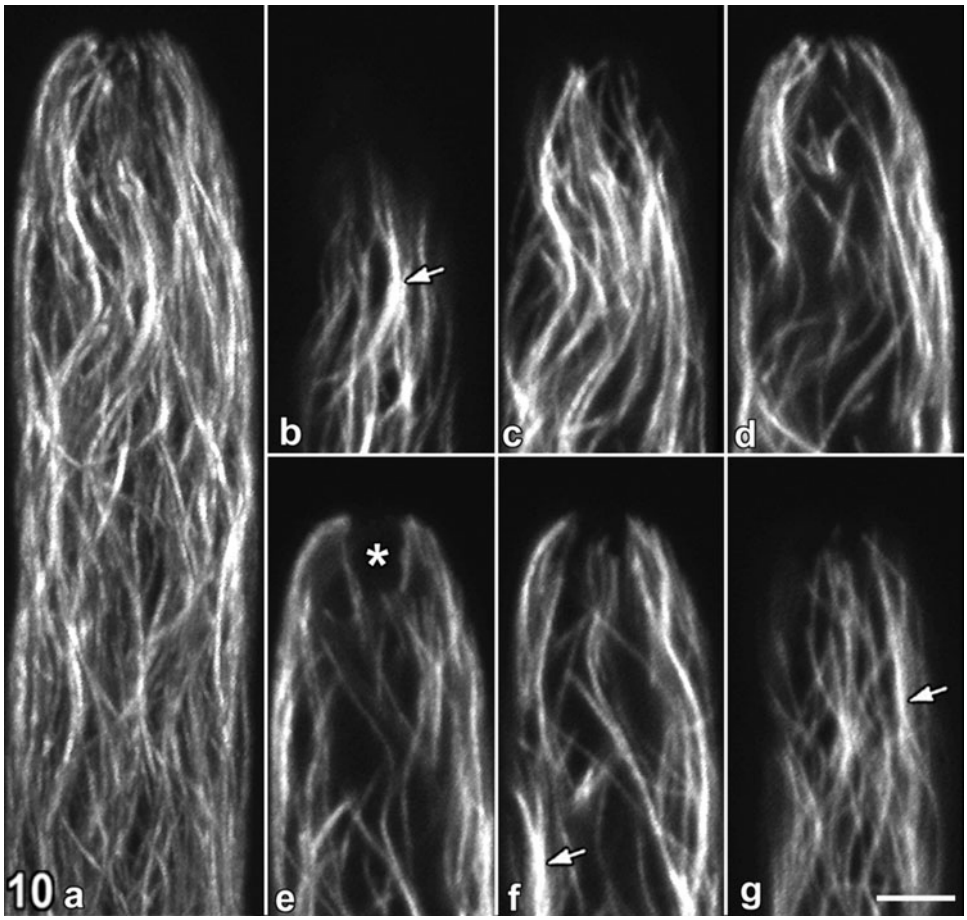

FIGURE 10 Immunofluorescence of MTs in hyphal cells of *S. rolfsii* visualized with confocal microscopy. (a) Reconstruction of six longitudinal optical sections through hypha. MTs are primarily oriented parallel to the growing hyphal axis exhibiting helical curvatures forming a loosely braided meshwork. (b through g) Optical serial sections. MTs are in close proximity to Spitzenkörper but at the time of cell fixation were not traversing Spitzenkörper (asterisk in panel e). MT bundles are indicated by arrows. Scale bar, 3 μm.

forming a left-handed helical filament. These short and flexible filaments are generally present in much higher numbers in the cytoplasm than MTs. Although actin is found throughout the cell, the highest density of actin filaments is in the cell cortex. The cortex is also the site for most MF nucleation. A single filament is about 7 nm in diameter and appears as a thin electron-opaque thread when viewed with TEM (Fig. 3). However, in fungal hyphae and other eukaryotic cells, actin MFs are visualized more often as complex networks and bundles. Like MTs, actin MFs are polar structures and are regulated through the interactions of many associated proteins.

Imaging of the actin cytoskeleton, including actin-associated proteins, reveals several distinct arrangements and distribution patterns in filamentous fungi. These include small spots or patches, longitudinal cables, and contractile rings associated with septum formation. At the LM level, the most obvious of these arrays are small cortical patches that are typically concentrated in a band located between 1 and 4 μm behind the growing tip of a mature hypha (Fig. 11) (Roberson, 1992; Srinivasan et al., 1996; Virag and Griffiths, 2004; Araujo-Bazán et al., 2008; Taheri-Talesh et al., 2008; Upadhyay and Shaw, 2008). Actin patches are excluded from the extreme hyphal tip and generally present in reduced numbers in the lower subapical hyphal areas. As noted previously, there is strong correlative evidence that filasomes are the ultrastructural equivalent of cortical actin patches. Araujo-Bazán and others (2008) have shown that endocytic proteins colocalize with actin cortical patches in mature hyphae of *A. nidulans*, supporting a spatially coupled mechanism of constitutive apical exocytosis and subapical endocytosis via filasomes/actin patches. In slower-growing germ tubes, the apical distribution of actin patches is less organized than in mature hyphae (Taheri-Talesh et al., 2008; Upadhyay and Shaw, 2008). In addition to cortical patches, a small apical aggregation or spot of actin has been reported in some fungi (Srinivasan et al., 1996; Taheri-Talesh et al., 2008) and corroborates EM data demonstrating a dense actin MF network associated with the Spitzenkörper (Fig. 3) (Bourett and Howard, 1991). In several fungi, proteins required for actin nucleation and organization (i.e., the polarisome) are found at the tips of growing hyphae (Knechtle et al., 2003; Harris et al., 2005) and some colocalize with the Spitzenkörper (Sharpless and Harris, 2002; Crampin et al., 2005).

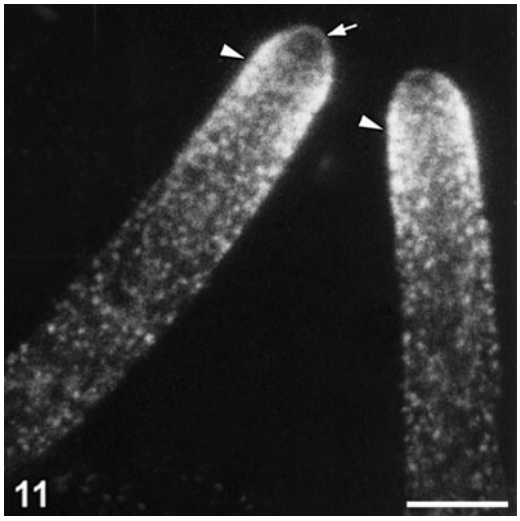

FIGURE 11 Immunofluorescence of actin in hyphal cells of *S. rolfsii* visualized with wide-field epifluorescence microscopy. Actin is localized as cortical patches in high numbers beneath apical domes (arrowheads). Actin spot was not observed at the apex (arrow) in these cells. Scale bar, 7 μm. Reprinted from *Mycologia* (Roberson, 1992) reproduced with permission from the publisher.

Septal Structure

Hyphae are broadly distinguished as aseptate or septate. Aseptate hyphae are found in members of several phyla of early diverging lineages, including zoosporic and zygosporic fungi. Even so-called aseptate hyphae, however, may be separated into active and nonfunctional portions by adventitious septa, i.e., septa that are not associated with mitotic divisions. Hyphae that are said to be regularly septate have septations that form in conjunction with nuclear divisions. Septate hyphae are divided into compartments that enable a division of labor as the hyphae mature from the growing tip backwards. Perforate septa allow for the selective passage of cytoplasm and some organelles between the compartments during their maturation. Below is presented a limited discussion of hyphal septation in the context of phylogeny and function.

Chytridiomycota, an early diverging zoosporic lineage of fungi, have adventitious septa that cut off parts of the often extensive mycelial system or rhizoids found in some species. Members of the traditional groups known as Zygomycota are usually considered to be coenocytic. Recent molecular analyses including additional taxa of zygospore-forming fungi, however, indicate that the phylum as previously defined may not be monophyletic (White et al., 2006; Hibbett et al., 2007). Certainly, a comparison of morphology, including septal characters, supports these organisms as more diverse than originally accepted. For example, one clade of zygospore-forming fungi lacks regularly septate hyphae but often has adventitious septa (White et al., 2006). Another large clade of zygospore-forming fungi, however, is characterized by regularly septate, plugged hyphae, and the characteristic plugs are used to distinguish among some orders within this clade (White et al., 2006).

Regularly septate hyphae are taxonomically important markers in Ascomycota and Basidiomycota, and these phyla often are referred to as septate fungi. A simple central septal pore (Fig. 12a) is generally present in hyphal members of the Ascomycota. However, there is significant structural diversity, especially among structures occluding the pores, for members of this phylum (AFTOL, 2007). Examples of such plugs in Pezizomycotina include nonmembranous material, granular lamellate structures, non-membrane-bound subspherical occlusions with translucent finger-like extensions from the pore margin, and non-membrane-bound subspherical occlusions without translucent finger-like extensions. In addition to the non-membrane-bound structures, membrane-bound Woronin bodies (Fig. 12a) (see "Woronin Bodies" below) may block the pores of more mature compartments in certain ascomycete (Pezizomycotina) hyphae (AFTOL, 2007).

The dolipore septum present in most Basidiomycota is characterized by an inflated region surrounding the septal pore (Fig. 12b) (Celio et al., 2006). In addition, within some taxonomic groups (Agaricomycotina) membranous septal pore caps (parenthosomes) and associated structures are positioned in the cytoplasm near the pore (Lutzoni et al., 2004). Septal pore caps are derived from the ER and appear as simple membranous caps or caps with extensions that may be cupulate, reticulate, or tubular. The extensions may be smooth vesicular-tubular membranes or multiple saccules; the cap may be reticulate. The cap also may be imperforate, uniperforate, or multiperforate with small or large pores and may have uniform electron-transparent or electron-dense contents with internal layers (AFTOL, 2007). The septal pore apparatus prevents the movement of large organelles (e.g., nuclei) from one compartment to another and is often associated with clamp connections. The septal pore system is less complex in other members of the Basidiomycota, including the earlier-diverging lineages.

Septal pore caps in *Rhizoctonia solani* (Basidiomycota) contain a glycoprotein that is associated with the ER (van Driel et al., 2008). This protein is also found in non-membrane-bound plugs that block the septal pore and, thus, are involved in regulating cellular activity at the pore.

Woronin Bodies

Woronin bodies are small (150 to 500 nm in diameter) electron-dense organelles that are spherical, oblong, hexagonal, or rectangular and bounded by a single-unit membrane (Fig. 12a) (Momany et al., 2002) (see chapter 16). Early evidence indicated that Woronin bodies functioned only as septal plugs to prevent loss of cytoplasm in damaged hyphae (Trinci and Collinge, 1974). More recently, Jedd and Chua (2000) demonstrated that the Woronin body is a specialized type of peroxisome. The size and number of Woronin bodies per cell are variable; a number of up to seven may be characteristic of certain species (Soundararajan et al., 2004). In the past, Woronin bodies were defined on the basis of morphology and position near the septal pore of ascomycetes. However, organelles with structural characteristics similar to those of Woronin bodies have been identified in conidia, appressoria, penetration pegs, and apical cytoplasm of germ tubes and hypha (Momany et al., 2002; Riquelme et al., 2002; Soundararajan et al., 2004; Hohmann-Marriott et al., 2006). In order to determine the identity of presumptive apical Woronin bodies, Momany (2002) and her colleagues used antibodies to the *N. crassa* Woronin body matrix protein, Hex1, to study the organelles in germ tube cells of *A. nidulans*. They provided evidence that the presumptive organelles in cell apices were in fact Woronin bodies that later were transported to the region of the developing septum.

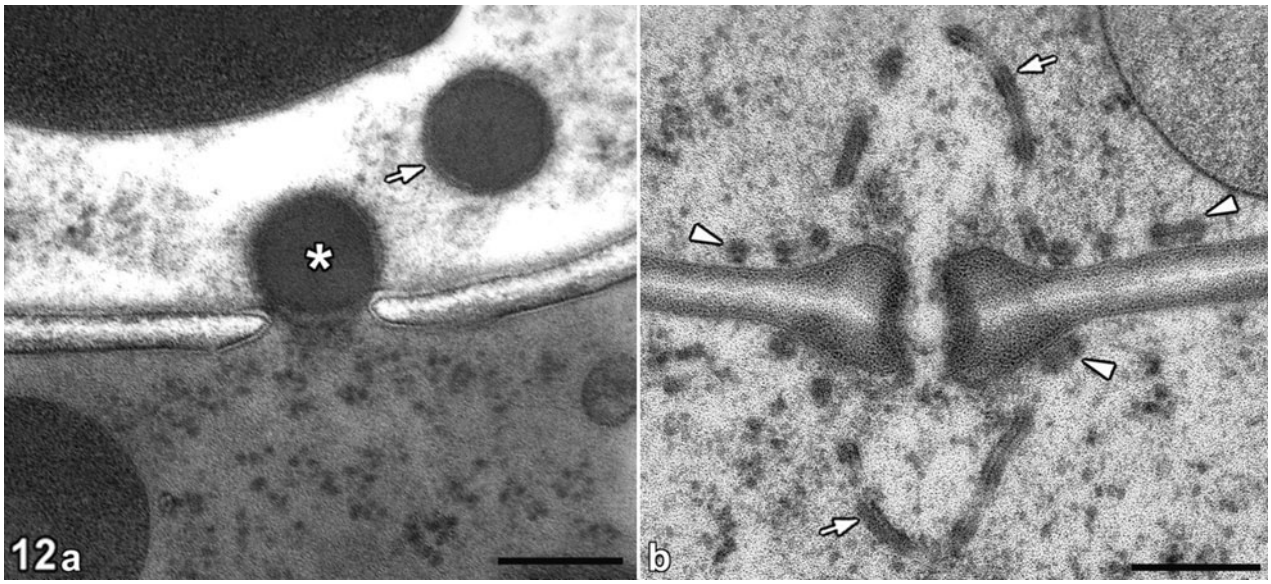

FIGURE 12 TEM of septal pore complexes. (a) Simple septal pore in a conidiation mutant of *Fusarium verticillioides* (Ascomycota). Woronin bodies are bound by a single-unit membrane (arrow). Note one of the Woronin bodies (asterisk) plugging the septal pore and the density difference between the cellular compartments above (apical) and below (distal) the cross wall. Scale bar, 300 nm. Courtesy of Beth Richardson and Tony Glenn. (b) Dolipore septal pore and septal pore cap (i.e., parenthosomes) (arrows) of *A. vulgare* (Basidiomycota) with the cap extending along the cross wall (arrowheads). Scale bar, 125 nm. Reprinted from *Mycologia* (Celio et al., 2007) with permission of the publisher.

APPLICATION OF CELLULAR STRUCTURE TO FUNGAL PHYLOGENETICS

Since the early 1960s the focus of some mycologists who acquire structural data has been to use selected cellular components as characters in taxonomic and evolutionary studies. Fine structure has been useful in distinguishing fungi from other organisms (Hawker, 1965; Heath, 1986; Sewall et al., 1989). More often, however, it has been applied to distinguish among fungal groups. Basidiomycete taxonomy benefited from early TEM studies of septa. The presence or absence of a dolipore septum provided the means of distinguishing single hyphae of ascomycetes and many basidiomycetes, and variation in septum structure provided new characters with taxonomic significance, especially in Agaricomycotina (McLaughlin et al., 1995; Celio et al., 2006). Characters derived from structures other than hyphae also have been important in mycology, and these include nuclear divisions used to distinguish some groups of fungus-like organisms from the monophyletic kingdom Fungi (for examples, see Heath, 1986; and Celio et al., 2006). TEM has been applied to fungi that cannot be cultured easily, including a number of obligate parasites of plants and animals, and has proved useful in some cases for observations on the morphology of very small fungi (Blackwell, 1994).

Newer methods to derive and analyze macromolecules have replaced structural fine detail in the minds of many systematists. Structure, however, remains important, especially when used in conjunction with sequence analyses that help to polarize evolutionary direction, a feat not usually possible with fine structure alone because the characters are often autapomorphic (i.e., confined to a single group). Characters shared by groups, synapomorphies, are needed to establish relationships.

SUMMARY

The ability to understand cytoplasmic structure can provide powerful insights into the biology of cells and organisms. This chapter has briefly reviewed the diversity of hyphal structures and presented examples of how bioimaging has contributed to a broader understanding of hyphal biology and phylogenetic relationships between fungal taxa. At the heart of polarized growth is the secretory pathway in which vesicles are targeted to sites of growth and subsequently fuse with the plasma membrane. In mature hyphae of the septate fungi, these events have given rise to the Spitzenkörper, a complex and dynamic structure that clearly influences hyphal growth and morphogenesis. Although there have been great advances in understanding the biology of the Spitzenkörper, profound questions still remain. For example, what are the unique characteristics of its three-dimensional structure, and are they applicable to phylogenetic analysis? What are the precise mechanisms of its function? Are the Spitzenkörper and polarisome one and the same? What are the processes regulating Spitzenkörper assembly and disassembly? These are complex questions that will require continued research using model organisms such as *A. nidulans*, *N. crassa*, and *Ashbya gossypii* (Ascomycota). However, it behooves us to apply our knowledge and efforts integratively and broadly so that a more complete understanding of hyphal growth among fungi can be realized. For example, what is the lineage of Spitzenkörper evolution? Are there specific advantages that the Spitzenkörper provides to growing septate hyphae that are absent from nonseptate hyphae? How common are Spitzenkörper in other, less-well studied taxa, such as hyphal members of the Chytridiomycota (e.g., *Spizellomyces punctatus* and *Monoblepharis insignis*) or zygosporic fungi

that manifest divergent characters towards the septate fungi (e.g., *Mortierella verticillata* [Mortierellales] and *Coemansia reversa* [Kickxellales])?

At the same time that some fungal biologists are asking questions about form and function, others are interested in the use of ultrastructural features as morphological traits that offer characters that support the results of molecular analyses in phylogenetic studies. The presence or absence of certain morphological characters (e.g., septa and Woronin bodies) already has been useful in defining higher taxa, especially since evolutionary polarity often can be established using stable phylogenetic trees based on DNA sequences. Ever-enlarging molecular databases, especially those of whole genomes, are allowing us to look for the genetic basis of many structural features, such as the presence or absence of Woronin body matrix proteins. This capability will allow us to understand the basis of these features not only in an evolutionary sense but also in a functional one. Collaboration among different types of fungal biologists including systematists is essential to understanding structure and how it applies to the study of the Fungi.

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3

A Top-Down Systems Biology Approach for the Identification of Targets for Fungal Strain and Process Development

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For many years, filamentous fungi have been used for the industrial production of a large variety of metabolites and proteins. A well-known example of a fungal bioprocess is the production of the secondary metabolite penicillin by *Penicillium chrysogenum*, developed about 60 years ago (Ligon, 2004). Fungal production processes for other β-lactam antibiotics, as well as drugs such as hypolipidemic agents (e.g., lovastatin by *Aspergillus terreus*) (Tobert, 2003), have been developed since. Furthermore, many of the commercial biological production processes for organic acids are fungal bioprocesses, including the production of citric, gluconic, and itaconic acid by *Aspergillus* species or lactic acid by *Rhizopus oryzae* (Magnuson and Lasure, 2004). Filamentous fungi also play an important role in the industrial production of proteins and enzymes. In particular, *Trichoderma* and *Aspergillus* species, but also *Penicillium* and *Rhizopus* species, are used to produce a large number of different enzymes, e.g., (hemi)cellulases, xylanases, chitinases, amylases, proteases, and many more (see the list of commercial enzymes from the Association of Manufacturers and Formulators of Enzyme Products [AMFEP] at www.amfep.org). The first industrial fungal bioprocess for proteins dates back even further than that for penicillin. For instance, the product takadiastase appeared on the market in 1894 and is in fact fungal amylase produced by *Aspergillus oryzae* (Gwynne and Devchand, 1992).

Some of the above-mentioned production processes have been developed and optimized over a period of decades, like penicillin, citric acid, and amylase; others have been developed more recently and are still being optimized to reach viable commercial production levels. This is particularly true for production of nonnative proteins by use of genetically engineered fungal strains. This chapter discusses approaches to select targets for improvement of production processes, with a special focus on the application of functional genomics technologies as an unbiased approach towards target selection.

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OPTIMIZATION OF FUNGAL PRODUCTION PROCESSES

The development of a fungal production process starts with the selection of a strain that produces the compound of interest or with the construction of such a strain. Once this strain is available, production levels need to be increased in order for the process to become economically viable. Optimization of the fungal production process, or any bioprocess for that matter, can be achieved by an iterative cycle of strain improvement and/or process optimization (Fig. 1). Process optimization includes improving medium performance as well as identifying optimal environmental process parameters, such as pH, temperature, and aeration. Many techniques are available for process optimization: straightforward methods like the change-one-factor-at-a-time approach or more advanced methods using the experimental design approach, for which various design and optimization techniques are available (Kennedy and Krouse, 1999; Weuster-Botz, 2000). Many of these techniques rely on prior knowledge of components and environmental parameters likely to affect product yields. This obviously means that many more components and parameters are overlooked that could be beneficial to bioprocess performance, but about which no prior knowledge is available. Similarly, strain optimizations until now mainly include alleviating bottlenecks identified in case-by-case studies. Often only the obvious targets for metabolic engineering are addressed (van der Werf, 2005). In the case of protein production, targeting known putative bottlenecks at the posttranscriptional stage is a commonly applied approach of optimizing production levels, for instance by alleviating blockages

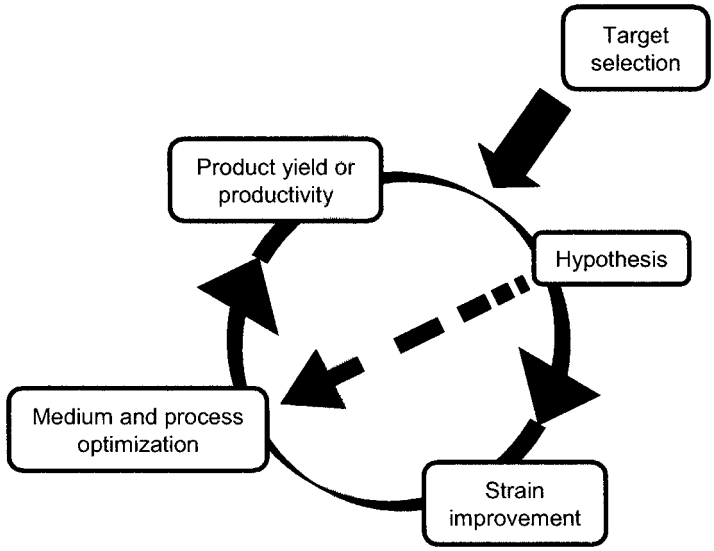


FIGURE 1 Iterative cycle of strain improvement and/or process optimization.

along the secretion pathway (Conesa et al., 2001) or by eliminating extracellular proteases (Braaksma and Punt, 2008). From the almost infinite number of genetic changes that can be introduced by overexpression or knocking out of genes, only those that are known from the current and generally limited knowledge of the metabolic pathway are selected to optimize product formation. Biological processes or interactions that are not currently known to be important for bioproduct formation or that are not yet known to exist are not taken into account.

In our research we aim at using a strain and process development approach which is not a priori hypothesis driven but relies on first acquiring data sets rich in information with regard to the bioprocess under study from functional genomics technologies and using these for target selection from the broadest possible ranges of expressed genes (transcriptomics), proteins (proteomics), or metabolites (metabolomics). In this chapter such a systems biology approach, based on the information gathered with functional genomics technologies and in combination with multivariate data analysis tools, is discussed as a method to achieve unbiased selection and ranking of targets for both strain improvement and bioprocess optimization.

TOP-DOWN SYSTEMS BIOLOGY

In systems biology the organism is studied as an integrated and interacting network of genes, proteins, and biochemical reactions. Principally, at its extreme, two approaches are recognized within systems biology: top-down and bottom-up systems biology (Bruggeman and Westerhoff, 2007). In bottom-up systems biology, biological knowledge is used as the starting point and a comprehensive mathematical model of the biological system under study is built. In fungal research metabolic stoichiometric or kinetic models and metabolic network topology models have been used for a systems-level investigation of mainly *P. chrysogenum* and *Aspergillus* species (David et al., 2006; Andersen et al., 2008a; Melzer et al., 2007; Gheshlaghi et al., 2007; Nasution et al., 2008). Similar to the more classical approaches for target selection, these methods require prior knowledge about the studied system. The models are built from known components only and demand an extensive knowledge of the individual parts of the model, and they exclude all components and reactions whose functions are not yet (fully) known.

In contrast, in top-down systems biology, data are used as the starting point and statistical data mining approaches are applied to come to a comprehensive understanding of the biological system. The principle behind top-down systems biology is that molecular components that respond similarly to changes in the experimental conditions are somehow functionally related. No other prior assumptions regarding the interactions of the studied molecular components are required. This allows the study of complex and relatively poorly characterized processes and strains, as extensive knowledge of the studied organism or process is not necessary. In this top-down systems biology approach there is also no a priori focus on specific biomolecules expected to relate to the biological question. Therefore, this approach also enables the discovery of previously unknown or unexpected relations between specific biomolecules and the biological process studied. Despite the potential of top-down systems biology, the great majority of scientists applying systems biology use a bottom-up systems biology approach. The reluctance towards top-down systems biology might relate to the risk of being overwhelmed by the enormous quantity of data that arise from functional genomics technologies such as metabolomics and transcriptomics. The challenge is to be able to extract relevant information from these data sets. Principally, the success of this approach depends on balancing three interlinked key factors: (i) definition of the biological question, (ii) experimental design, and (iii) the data analysis tool (Fig. 2). These three factors are discussed in more detail below.

THE BIOLOGICAL QUESTION

A clear definition of the biological question to be answered is the crucial starting point in any top-down systems biology research project, because only then can a suitable experimental setup and data analysis strategy be selected (van der

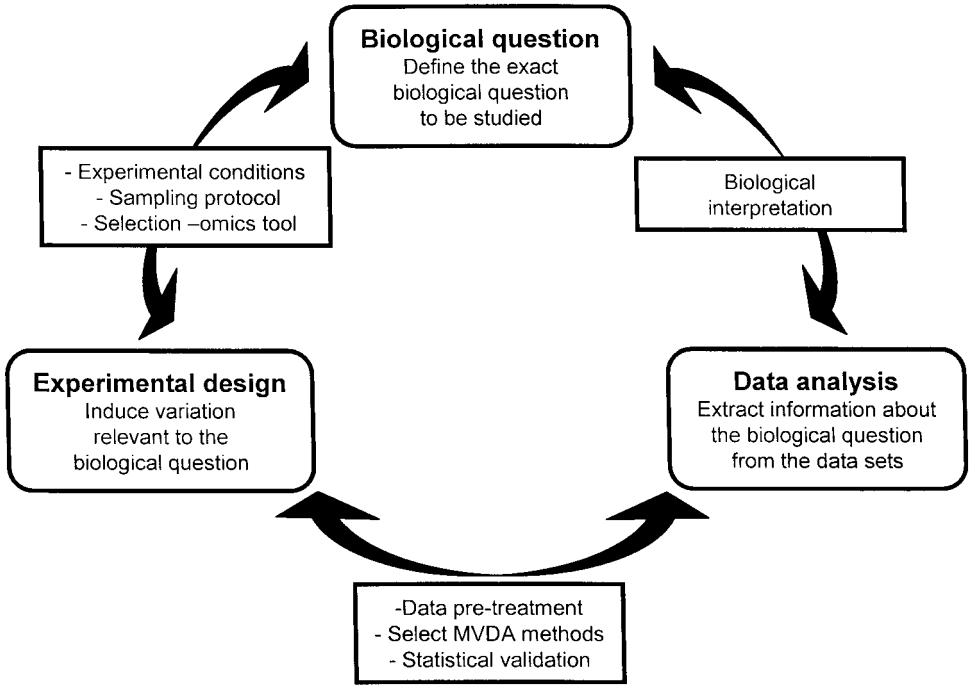


FIGURE 2 Key conditions and their relation to a successful systems biology study. In top-down systems biology, three interlinked factors are crucial for success: (i) the biological question, (ii) the experimental design, and (iii) data analysis.

Werf et al., 2005; Trygg et al., 2007). To explain this in more practical terms, two examples are given of ways to define the biological question in a study to gain more insight in the regulation of the proteolytic system of *Aspergillus niger*. First, when this problem is approached on a metabolic level, the biological question could be, "Which metabolites induce protease activity in *A. niger*?" On the other hand, when this problem is approached on a genetic level the biological question could be stated as, "Which transcriptional regulators are associated with protease activity in *A. niger*?" In the first case metabolite levels are the relevant biomolecules to be measured, in the second case transcript levels are to be determined, and in both cases protease activities will have to be determined. What is important is that the biological question be translated into a quantifiable biomolecule level, which can be measured at different biochemical levels (i.e., at the transcriptome, metabolome, or proteome level). In addition, it is often possible to specify a quantifiable phenotype that is relevant for the biological question, such as protease activity in this case. It is also very important to clearly define this phenotype. For instance, in the production of a biological compound or activity, among others, the following definitions of phenotypes could be chosen for improvement: concentration (in grams per liter) or activity (in units per liter); specific concentration or activity (in grams per gram dry cell weight or in units per gram dry cell weight); productivity (in grams per liter per hour or in units per liter per hour); specific productivity (in grams per gram dry cell weight per hour or in units per gram dry cell weight per hour). When reducing costs of nutrients is the key goal, one could also think of defining the phenotype as cost of nutrients per unit product (in U.S. dollars per gram of product) or cost of nutrients per unit productivity (in U.S. dollars per gram of product formed per liter per hour) (Kennedy and Krouse, 1999). The biological question and its translation into a practical format strongly influence the other key factors of a top-down systems biology study, i.e., experimental design and data analysis. The experimental setup should ensure that experimental conditions that induce variation relevant for the biological question are selected and that data analysis is able to extract the information relevant to the biological question from the functional genomics data set.

EXPERIMENTAL DESIGN

Based on the biological question, the experimental design of the top-down systems biology study should be aimed at generating large information-rich data sets in order for data analysis to extract relevant biological information from the data set. Not only experimental conditions for the experimental design should be considered, but also sampling, sample workup, and the functional genomics tool to be used to analyze the samples.

Experimental Conditions

The first step in establishing how to plan and conduct the experiments is to identify those parameters affecting the response of the phenotype. These parameters can be process type (batch, fed-batch, or continuous), environmental conditions such as pH and nutrients, or selected strains. In the case of using various mutant strains to induce variation in the data set (for an example, see Askenazi et al., 2003), one should keep in mind that each strain may have its own bottleneck, making identification of specific targets for a general improvement more complex. When a phenotype relevant to the biological question is available, the experimental conditions should be targeted to induce variation in

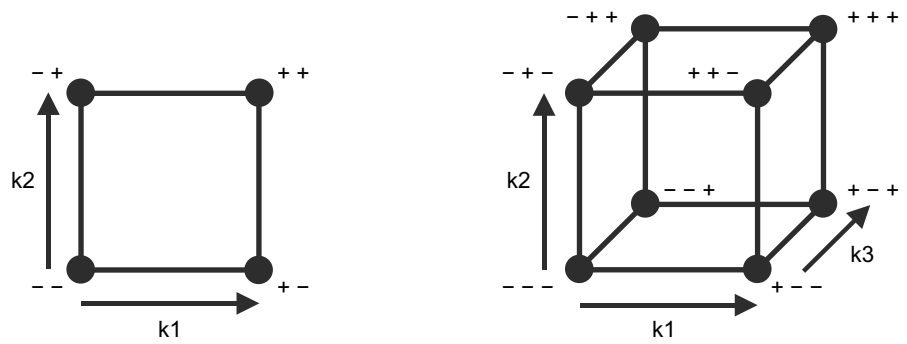
this phenotype. When it is unclear what experimental factors are involved in the induction of biological variation relevant to the biological problem, screening experiments need to be conducted to obtain more information regarding these experimental factors.

Traditionally, one of the most frequently used approaches to study which parameters affect biological responses is the change-one-factor-at-a-time approach, in which one independent variable is studied while all others are fixed at a specific level. An advantage of this simple and easy method is that any change in response can be attributed to a specific change. On the other hand, this change-one-factor-at-a-time approach has some serious drawbacks, perhaps the most important being that possible interactions between components are ignored. As a result, this approach frequently fails to find optimal conditions for experiments. Another disadvantage is the unnecessarily large number of experiments that are required when testing more than a few variables. Therefore, the change-one-factor-at-a-time method is acknowledged to have severe shortcomings and is more and more being replaced by statistics-based experimental designs, also called "Design of Experiments." For an initial screening of factors possibly related to the biological question, different types of experimental designs, so-called screening designs, are available, including the full factorial design (Lundstedt et al., 1998). In a full factorial design, every level of a factor is investigated at all levels of all other factors. Often the factors are investigated at two levels, requiring a number of runs equal to 2^k for k factors, which results in a large number of experiments when many factors are investigated (Fig. 3). When the factors are investigated at three or more levels, requiring 3^k runs in the case of three levels and n^k runs for n levels, the number of experiments rapidly becomes impracticable. To reduce the number of experiments without the loss of too much information, several experimental designs derived from the full factorial design are available. The most commonly used one is the fractional factorial design (Lundstedt et al., 1998; Trygg et al., 2006), which requires only n^{k-p} number of runs, with k as the number of investigated factors at n different levels, and p describing the size of the fraction of the full factorial used. With this type of design, three-way and higher interactions are ignored. Another useful screening tool is the Plackett-Burman design (Plackett and Burman, 1946; Weuster-Botz, 2000). This experimental design is a variation on the fractional factorial design, but instead of ignoring only higher interactions it considers all interactions between factors negligible. The downside of the last two designs is that when interactions between factors are not negligible, they are confounded with the estimated effects. This means that the estimated effects and those interaction effects cannot be distinguished from one another.

Based on this first phase, the main factors relevant to the biological question under study are selected for the final setup of experiments for the top-down systems biology study. In principle, statistical experimental designs for this phase can be any of the methods as described above. While in the screening phase the goal was to find out a little about many factors, in this phase the goal is to extract the maximum amount of information from the experiments, preferably in the fewest number of runs. Types of statistical experimental designs suitable for this phase of the study include central composite designs and Box-Behnken designs, which are both based on (fractional) factorial designs, or D-optimal designs, a computer-aided design method (Kennedy and Krouse, 1999; Trygg et al., 2006; Lundstedt et al., 1998). On top of that, response surface methodology can be applied to generate a data set with an evenly distributed variation. Response surface methodology is commonly used in industry for process optimization (Dobrev et al., 2007; Li et al., 2007). Based on a set of designed experiments, e.g., from a factorial design, a model that predicts the biological response to different levels of the various factors included in the study is built. In contrast, from such a model, conditions that will result in various levels of relevant biological response can be selected for the top-down systems biology study.

Selection of a Functional Genomics Tool

Selection of the functional genomics tool to be used in a top-down systems biology study depends on the level at which the biological phenomena relevant for the biological question occur. With transcriptomics the expression levels of mRNA under a given condition are examined. The transcriptome reacts very fast, within a few minutes, to environmental changes. This makes transcriptomics a very suitable tool to study the cell exposed to changing environmental conditions, such as the addition of toxic or chemical compounds (Arvas et al., 2006; Guillemette et al., 2007) or transfer from one medium to another (Yuan et al., 2006). However, mRNA levels do not directly correlate to the levels of the encoded protein, due to posttranscriptional regulation steps at the level of mRNA stability, processing, and



A. 2^2 full factorial design **B**. 2^3 full factorial design

FIGURE 3 Full factorial designs, with two factors (A) or three factors (B) investigated at two different levels.

translation. Therefore, transcriptomics is only an indirect approach to study the function of a cell. On the other hand, the proteome and the metabolome together determine the actual function of the cell (the phenotype) (Oliver, 2000).

The proteome, meaning all proteins present at a given moment under defined environmental conditions, gives an indication of which metabolic pathways occur under those conditions (Kim et al., 2007a), as many proteins are enzymes that catalyze biochemical reactions. In contrast to transcriptomics, quantitative proteomics is still far from being a comprehensive analysis tool, mainly due to the limited dynamic detection range and poor reproducibility of proteomic analysis. Because of this there is a very strong bias towards identifying only the more abundant proteins in a complex proteome sample. Nonetheless, to study posttranslational modifications of proteins, such as phosphorylation and glycosylation, proteomics is the most obvious tool (Fryksdale et al., 2002; Kim et al., 2007b).

The metabolome of the cell, i.e., all metabolites present in a cell at a certain moment, provides valuable information about the regulatory or catalytic properties of either mRNA or enzyme, as metabolites are downstream of all genome and proteome regulatory structures (Oldiges et al., 2007). As the metabolome is closest to the phenotype of a cell, it will be most relevant in order to understand biological functioning. Similar to what was noted above for proteomics, full coverage of the complete metabolome is not (yet) accomplished by the available analytical platforms, although some metabolomics platforms are approaching the ultimate goal of providing a universal platform for the comprehensive and quantitative analysis of microbial metabolomes (van der Werf et al., 2007).

Sampling Strategy

The sampling strategy is part of the experimental setup and describes when and how samples for the functional genomics analysis are collected. It embraces two main issues, namely, collecting the sample at a time point where the biological response relevant to the biological question is present and ensuring that levels of biomolecules remain unchanged from the moment of sampling. Concerning the first issue, if it is unknown beforehand which phases during the cultivation contain information related to the biological question, the sampling protocol should cover all possibly relevant growth phases and phase transitions (Trygg et al., 2007). At the same time, practical matters have to be considered as well. For instance, the sampling volumes can limit the number of obtainable samples, or the costs of sample analysis can influence the sampling strategy. In the case of continuous cultures, time issues are of no importance, but due to technical difficulties this fermentation technique is not as commonly applied in fungal research as it is in research involving other microorganisms. Besides, with the application of continuous cultures the approach is quite different, as time is no longer a factor, excluding longitudinal effects (e.g., induction or other perturbations during the fermentation process). In addition, it should be noted that although the process conditions are fixed during continuous cultures, changes in the production organism are frequently observed (Swift et al., 1998; Withers et al., 1995), making continuous cultures prone to transitions, albeit of a different kind.

The second issue relates to the high turnover of mRNA and metabolites (for proteins this is not so much of an issue), risking the introduction of unwanted changes in RNA or metabolite levels during sample harvesting or workup. In order to obtain samples that reflect the state of the cell under the environmental conditions at the time of harvesting, rapid sampling (Nasution et al., 2006) and immediate inactivation (quenching) of the cellular metabolism are a necessity. In the literature, the quenching methods used for filamentous fungi mainly include rapid filtration followed by immediate freezing of the cells (David et al., 2006) (mostly used for transcriptomics samples) or dilution of the cells in a methanol solution of −45°C (more often used for metabolomics samples) (Ruijter and Visser, 1996; Nasution et al., 2006; Kouskoumvekaki et al., 2008).

After quenching the cells, conditions should be maintained during sample workup in order to prevent changes in the metabolite composition or RNA levels due to residual enzymatic activity present in the samples. Extraction of RNA from mycelia is often accomplished by disruption of the cells by either grinding under liquid nitrogen with a mortar and pestle (Kimura et al., 2008; Foreman et al., 2003) or bead milling at temperatures of approximately 4°C (Andersen et al., 2008b), followed by a standard RNA isolation protocol. Extraction of proteins is done in a similar way, without the stringent control of temperature (Carberry et al., 2006). For fungal metabolomics samples, two methods in particular have been described for extracting metabolites from the cells. The first is boiling the cells in an ethanol-buffer solution and subsequent reduction of the volume by evaporation in a rotavapor (Nasution et al., 2006). The second is chloroform extraction at −45°C (Ruijter and Visser, 1996).

A final issue to consider as part of the sampling strategy is replicates. As the total variation in the data set is the sum of technical, uninduced biological, and induced biological variations, repeated measurements may be necessary to estimate the individual contributions of these various parts. However, in general the biological variation is much larger than the variation induced by sample workup or variation in the analytical method (van den Berg et al., 2006). This makes repeating the experimental procedure with identical samples not very worthwhile in most cases. Some biological replicates will have to be included in the experimental design to estimate the overall uninduced biological variation due to small differences between biological conditions or biological variability. In this way, the induced biological variation can be established, calculated on the basis of the differences between the experimental conditions.

Based on the various aspects of the experimental setup discussed above, it becomes clear that it is necessary to balance the demands from the biological question and the data analysis on one side with practical considerations on the other.

DATA ANALYSIS

After having generated data sets under several different conditions with hundreds or thousands of proteins, mRNAs, or metabolites, the remaining challenge is to extract information about the biological question from these enormous data sets. Multivariate data analysis (MVDA) tools are preferably used, as those tools take into consideration the intrinsic interdependency of the biomolecules. But before the data sets can be analyzed by MVDA tools, the data output from the various functional genomics methods often requires data pretreatment.

Data Pretreatment Methods

In addition to the specific preprocessing steps of the data output from the various genomic methods, such as deconvolution of data files generated by gas chromatography-mass spectrometry for metabolomics (van der Werf et al., 2005) or normalization of cDNA microarrays (Leung and Cavalieri, 2003), another critical step before applying MVDA tools is data pre-

treatment of the data sets. Data pretreatment procedures correct for the influence of factors such as the abundance of a biomolecule or the magnitude of the change, which are generally not a reflection of the importance of a biomolecule (van den Berg et al., 2006). Appropriate data pretreatment methods will articulate the *biological* information content and will consequently allow more relevant biological interpretation of the data set. Three classes of data pretreatment methods can be distinguished: centering, scaling, and transformation. The last two methods are always applied in combination with centering. In MVDA, mean centering and autoscaling are the two most commonly used data pretreatment methods. With mean centering, the average level of a biomolecule is subtracted from each individual experiment, thereby adjusting for differences in the offset between high-abundance and low-abundance biomolecules. With autoscaling, the values are subsequently divided by the standard deviation of each biomolecule, adjusting for disparities in increase/decrease differences between the various biomolecules. In addition to these two methods, range scaling holds great promise, as the mean centered values are not divided by a statistical measure for data spread, as is the case with autoscaling, but by a biological measure, namely, the biological range. The biological range is the difference between the minimal and maximal levels reached by a certain biomolecule in a set of experiments. In Fig. 4 the effect of data pretreatment on principal component analysis (PCA) results of a metabolomics data set of *Trichoderma reesei* is shown (van der Werf et al., unpublished data).

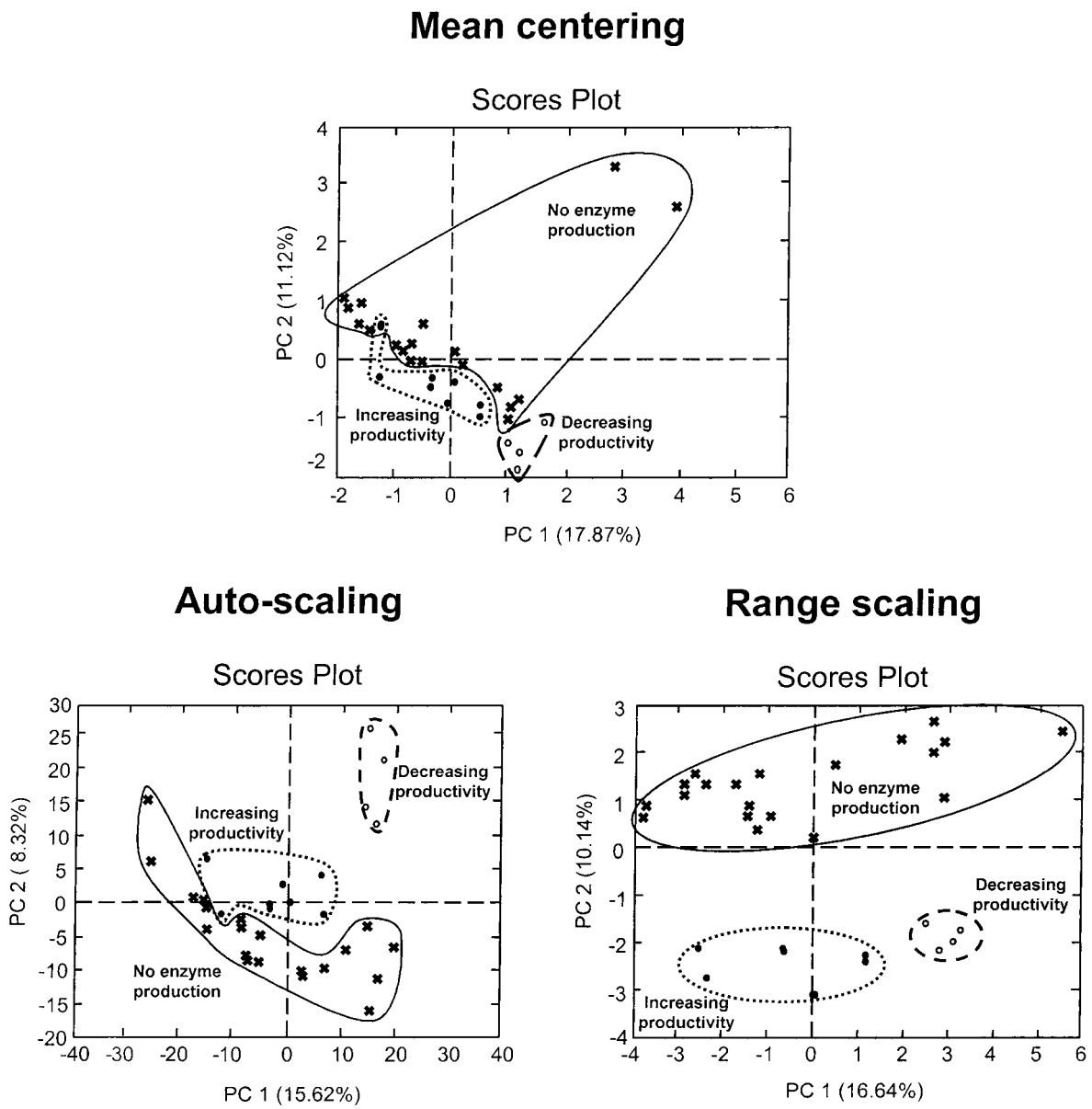


FIGURE 4 The effect of mean scaling, autoscaling, or range scaling of metabolomics data sets on PCA data results. The data sets are derived from research related to induction of cellulase activity in *T. reesei* (van der Werf et al., unpublished data). The metabolomes of three groups of samples (no enzyme production, increasing productivity, and decreasing productivity) were analyzed and pretreated with these three different approaches and subsequently analyzed by PCA.

With data pretreatment the biological information content in the data set is accentuated. In this particular case, it is range scaling that especially emphasizes the biological variation among the different biological groups. This data pretreatment method allows a clear separation of these different groups, whereas no grouping or a less obvious grouping is observed in the data sets when the other two methods are used.

MVDA Tools

Choices in data analysis strategy are influenced by the biological question, the characteristics of the experimental design, the behavior of the relevant biomolecules, and the dimensions of the data set. There are various MVDA methods that address different biological questions. In general, these methods can be divided in two main groups, namely, unsupervised methods and supervised methods. Unsupervised methods include PCA (Jackson, 1991; Jolliffe, 2002) or hierarchical clustering analysis (Eisen et al., 1998) that visualize relations/patterns in data sets without prior knowledge. Supervised methods, which include regression methods such as partial least squares (Geladi and Kowalski, 1986) and principal component regression (Mardia et al., 1979) or classification methods such as partial least squares discriminant analysis (Barker and Rayens, 2003) and principal component discriminant analysis (Hoogerbrugge et al., 1983) do the same as unsupervised methods while at the same time prior knowledge about one or more biological properties of the data set are taken into consideration. Discriminant methods are particularly suitable for samples with no quantifiable phenotype other than the presence or absence of a certain biological characteristic, e.g., morphological traits such as color or hyperbranching or certain environmental conditions or perturbations. For discriminant methods, this means that the samples are divided in (biological) groups, e.g., a group of samples from the wild-type strain and a group of samples from a mutant. Although each sample within such a biological group is designated as equal, there will always be biomolecules correlating to specific groups that are irrelevant to the biological question under study (so-called chance correlations). Therefore, when it is possible to express the phenotype as a numerical figure, this is preferred, as the risk of chance correlations is reduced when analyzing such data with regression methods. Regression methods find correlations between a numerical phenotype response and the biomolecule composition for the different samples in the data set. Regression methods are preferably applied to a set of experiments with large and evenly distributed variation in the biological response of interest.

In addition, validation of the data analysis results is of crucial importance, as it will provide an indication of the risk that correlations were found by chance due to the relatively low number of samples in relation to the large number of measured biomolecules. As multivariate statistical methods were developed for data sets containing many samples and few variables, this is a serious risk. Frequently applied data analysis validation strategies in top-down systems biology are cross validation, permutation, jackknifing, and bootstrapping (Rubingh et al., 2006; Westerhuis et al., 2008; Efron and Tibshirani, 1993). Based on the results of these validation steps, the reliability of the obtained models is established. Finally, a list of biomolecules can be obtained with the largest contribution to the model, i.e., those with the highest absolute regression factor. The biomolecules with the highest ranking are considered to be most relevant to the studied biological phenomenon.

Biological Interpretation

Based on the list of biomolecules identified by the MVDA tools as being important in relation to the question under study, targets for improvement of the production process have to be selected. There is a possibility with MVDA tools that biomolecules that do not show an unambiguous interaction with the specific biological question will be identified. Therefore, one of the first steps is to go back to the original data sets and examine fluctuation of the concentration of the biomolecule in relation to the studied phenotype. Moreover, not all biomolecules that exhibit an apparently strong interaction with the studied phenotype are biologically related to it. For that reason, as much information as possible should be acquired about the biological function of these biomolecules in the context of the question under study. From this knowledge, biological hypotheses will have to be formulated and new experiments will have to be set up to test them. For targets from transcriptomics studies, this can be quite straightforward, by either overexpression or deletion of the designated relevant genes, depending on a positive or negative correlation with the phenotype. On the other hand, several options for the ultimate improvement of the process are possible for targets identified in metabolomics studies. An easy way to increase product levels might be the addition or omission to the growth medium of a relevant metabolite identified by data analysis. This approach bears the risk that the transport of the compound into the cell will limit its suitability. More complex is the segue from a relevant metabolite identified by using metabolomics to a gene target for metabolic engineering. This requires knowledge about the metabolic pathway(s) involving the metabolite and its putative (allosteric) regulatory effects. Even then, it is not straightforward to translate this knowledge into a gene target. For instance, when a positive correlation between the product of interest and an intermediate in the biosynthesis route for the product is observed (an increase in the concentration of this intermediate correlates with elevated product levels), the enzyme converting the intermediate is not active enough and the corresponding gene should therefore be overexpressed. In another example, elevated product levels correlate with increased levels of an intermediate via a side reaction. Elimination of this competitive pathway by deletion of the corresponding gene should result in an increased flux through the biosynthetic pathway of interest and thus elevated levels of the desired product.

RESEARCH EXAMPLE: A TOP-DOWN SYSTEMS BIOLOGY APPROACH TO STUDY PROTEASE INDUCTION IN *A. NIGER*

At the Microbial Production Processes group of TNO Quality of Life, part of the internationally operating Netherlands Organization for Applied Scientific Research, filamentous fungi are developed as production hosts for heterologous proteins. For this application of filamentous fungi, degradation by proteases is acknowledged to be one of the key factors limiting heterologous protein yields. Besides classical approaches for strain development, such as screening for protease mutants and targeted disruption of known protease genes (Braaksma and Punt, 2008), a top-down systems biology approach was applied to further elucidate the proteolytic system and its regulation in *A. niger*. The ultimate goal is to identify new targets for further improvement of the fungal cell factory for heterologous protein production.

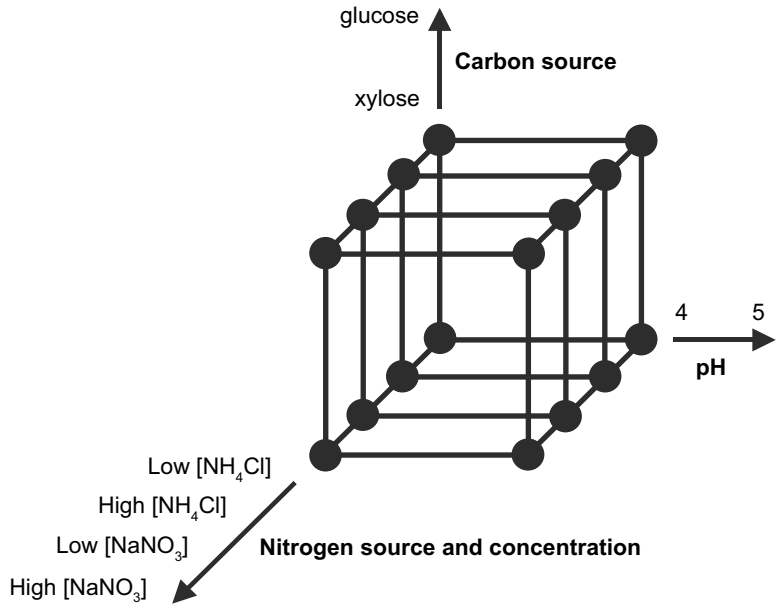


FIGURE 5 Full factorial design of the experiments for the top-down systems biology approach to study the regulation of the proteolytic system of *A. niger*. Four factors were varied at two different levels.

Approach

During the past decade, an advanced analytical platform has been developed at TNO that allows the comprehensive analysis of a broad range of metabolites (van der Werf et al., 2007). This metabolomics platform has been successfully applied for metabolomics studies of various microorganisms, including a study for metabolites linked to cellulase induction in *T. reesei* (van der Werf et al., unpublished data). This platform was therefore used to study the regulation of the proteolytic system of *A. niger*, with the specific biological question: "Which metabolites induce protease expression in *A. niger*?" In order to perform a top-down systems biology study, data sets in which the levels of protease activity varied were required. To this end, after an initial screening of conditions influencing protease activity in *A. niger* cultures, a series of experiments were performed according to a full factorial design involving four factors that were investigated at two levels (Braaksma et al., 2009). The four factors included were pH, carbon source, nitrogen source, and nitrogen concentration (Fig. 5). At different phases during growth, samples were collected and prepared for metabolome analysis (Braaksma et al., unpublished data). As it was not clear beforehand how to define the phenotype protease activity, several definitions were used and compared during the course of the research (Fig. 6). In the case where bioproduct formation occurs during the stationary phase, as is observed with protease activity, the specific activity, concentration, or productivity could be calculated from the actual biomass or the maximum biomass. Due to lysis, biomass levels might decrease significantly during the stationary phase, resulting in deceivingly high values for specific phenotypes, or further increase even when actual production has ceased.

The following example illustrates that having a well-thought-out experimental design beforehand (including the choice of functional genomics tools and sampling strategy) is crucial for a successful top-down system biology study. After the culturing experiments had been performed, the opportunity occurred to also perform transcriptomics analysis with a limited number of samples. This allowed investigation of transcriptional changes that are associated with protease expression in *A. niger*. As the experiments were not designed specifically for transcriptional analysis, the selection of samples to be used for RNA extraction and microarray analyses turned out to be the critical step. The response of transcript levels to environmental changes is preceded by regulation by metabolites, which make sampling at specific time points even more crucial for transcriptomics. The earliest samples in which protease activity was detectable were selected for the transcriptomics analyses. Due to this criterion, some of the selected samples were from a time point at which the carbon source was still present in the medium, while another set of samples were from times when the carbon source had been depleted. Unexpectedly, statistical data analysis of the results from the microarray revealed that variation in protease activity due to various growth conditions was actually dominated by variation due to the different biological time points of the samples. PCA showed that instead of clustering samples together based on similar protease levels, samples clustered based on whether the carbon source was still present or not. Knowledge not only of protease activity as phenotype but also of other measured culture parameters allowed identification of this effect. Based on these results, it was clear that additional microarray experiments using an experimental design with the explicit intent of applying transcriptomics would be preferred to study transcriptional changes associated with protease expression in *A. niger*.

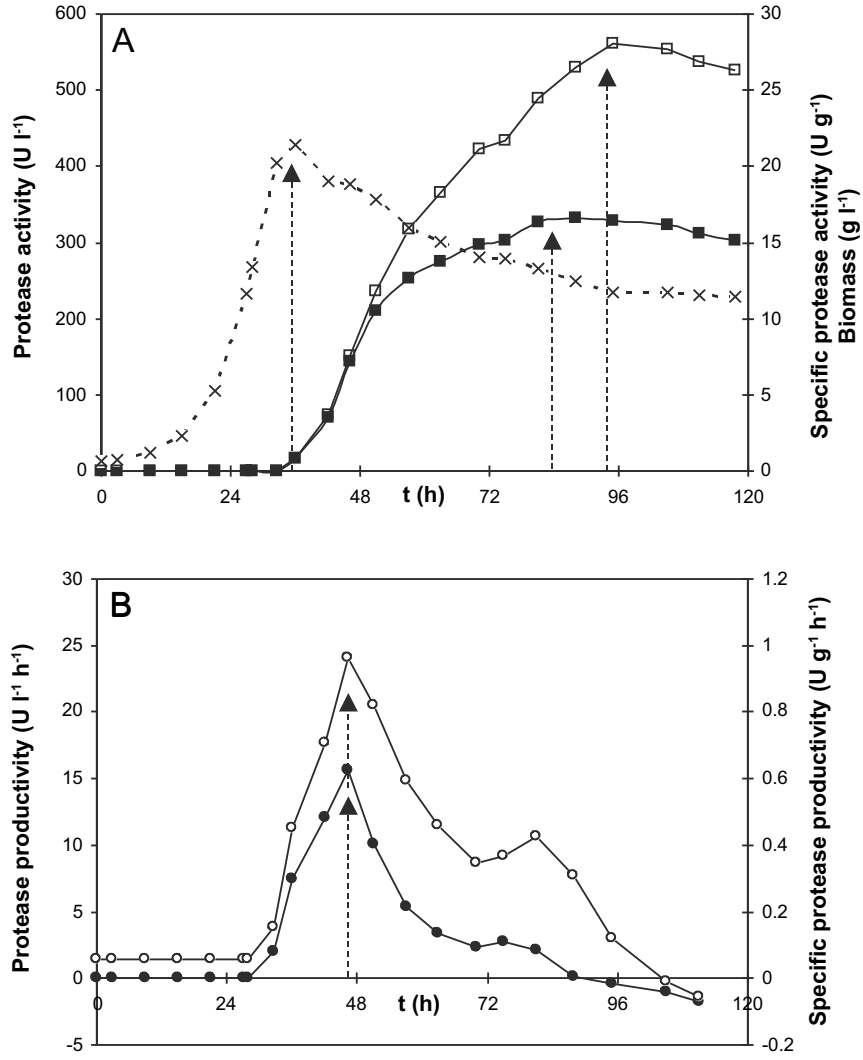


FIGURE 6 Representation of different protease phenotypes during a glucose-based batch culture. (A) Protease activity (■) and specific protease activity (□). (B) Protease productivity (●) and specific protease productivity (○). Time points at which the biomass (×) and each different phenotype reached its maximum value are indicated by upward arrows.

CONCLUSIONS

The available selection methods for relevant targets for fungal strain and process development, or for that matter any microbial production process, have been very successful in numerous cases. However, the exclusion of all biological processes or interactions that are not currently known to exist has been shown to hamper further improvement while using these approaches. Recently introduced functional genomics technologies in combination with MVDA tools enable an open and comprehensive top-down systems biology approach towards target selection. Nevertheless, the success of such an approach depends heavily on a systematic study covering all aspects, from a clear description of the biological question up to statistical data analysis. As this involves knowledge beyond the biologist expertise (e.g., biostatistics), the assistance of experts in other fields will be indispensable. Due to its unbiased nature, a successful top-down systems biology approach will provide a new boost in the ongoing cycle of bioprocess optimization.

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4

Phylogenetics and Phylogenomics of the Fungal Tree of Life

JOSEPH W. SPATAFORA AND BARBARA ROBBERTSE

The past 2 to 3 decades have witnessed both the birth of molecular phylogenetics of fungi and its development into a fundamental tool for comparative biology of fungi. Phylogenetic trees, once restricted to studies on systematics, are now used throughout all disciplines of fungal biology and provide the evolutionary context for a broad suite of studies that include understanding the evolution of major life forms, description of complex biotic communities, and predictive experimental biology. This is especially true in the genomic era, where a rapid convergence of phylogenetics and genomics is occurring and is resulting in the emerging field of phylogenomics. Although a young field, phylogenomics has already garnered multiple definitions including the use of evolutionary relationships to predict homology and function of uncharacterized genes, and the construction of phylogenies based on genome-scale sampling of the maximum—or at least a large amount—of discrete orthologous sequence data. Here we provide a review of (i) the current status of fungal phylogenetics based on multigene phylogenies, (ii) current evolutionary hypotheses on the evolutionary relationships of organisms that are classified in the Kingdom Fungi, and (iii) the use of genome-scale sampling to infer evolutionary relationships of the fungi.

MOLECULAR SYSTEMATICS OF THE FUNGI

Since the first molecular phylogenetic studies of the Kingdom Fungi, rRNA genes have played a prominent role in inferring phylogenetic relationships of the kingdom (Bruns et al., 1992). This is largely based on the fact that rRNA genes are ubiquitously distributed across life and that these genes possess regions of nucleotide conservation, which allowed for the development of universal primers (Bruns et al., 1991). Thus, the collection and alignment of rRNA nucleotide data were relatively straightforward and resulted in an exponential increase of molecular phylogenetic studies of the Fungi throughout the 1990s (Lutzoni et al., 2004). Although these analyses were collectively based on a small set of data, numerous landmark discoveries that greatly advanced the field of fungal phylogenetics were made regarding the evolution of fungi and fungus-like organisms. These include, but are not limited to (i) the placement of the heterokont water molds (Oomycota) and slime molds (Myxomycota) outside the Fungi (Gunderson et al., 1987; Baldauf and Doolittle, 1997); (ii) recognition of a close evolutionary relationship between the Animal Kingdom and Fungi (Baldauf and Palmer, 1993); (iii) defining a monophyletic Fungi to include Chytridiomycota, Zygomycota, Basidiomycota, and Ascomycota (reviewed by Blackwell et al., 2006); (iv) characterization of the Chytridiomycota as comprising the early-diverging lineages of Fungi (James et al., 2000); (v) recognition of the arbuscular mycorrhizae (Glomeromycota) as a taxon distinct from the Zygomycota (Schüβler et al., 2001); and (vi) support for the monophyly of Ascomycota and Basidiomycota and their sister group relationship (Lutzoni et al., 2004).

In spite of these advancements, it became clear that rRNA data alone were limited in their utility and that many important phylogenetic questions pertinent to understanding the evolution of the Fungi would require additional genes and likely different types of genes, i.e., protein-coding genes (Liu and Hall, 2004; Lutzoni et al., 2004; Reeb et al., 2004). PCR and sequencing primers with fairly broad applicability were developed for several nuclear protein-encoding genes, with the first and second largest subunits of RNA polymerase II (*RPB1* and *RPB2*, respectively) and translation elongation factor 1-alpha (*TEF*) being the most widely used protein-coding genes in fungal systematics. These genes provided independent assessments of rRNA phylogenies and resulted in more robust tests of morphological and ecological evolution of the Fungi (Blackwell et al., 2006). These genes also provided the raw data that initiated the production of multigene phylogenies and arguably transformed the field of fungal phylogenetics from gene trees to species trees.

In order to advance the development of multigene phylogenies, the fungal systematics community created the Research Coordination Network Deep Hypha (Blackwell et al., 2006). The goal of the Deep Hypha Research Coordination Network was to accelerate the collection of multigene

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sequence data across the Fungal Tree of Life. This was achieved by the creation of the community-based "Assembling the Fungal Tree of Life" (AFTOL) Project, which promoted the collection of nucleotide sequence data for six loci—nuclear small subunit rRNA and nuclear large subunit rRNA, mitochondrial small subunit rRNA, *RPB1*, *RPB2*, and *TEF*—for a target set of taxa across the major orders and families of the Fungi (Lutzoni et al., 2004). AFTOL resulted in the collection of more than 5,000 publicly available sequences for more than 2,000 taxa and the development of additional primers for enhanced data collection across the Fungi. (Visit www.aftol.org for a complete list of sequences and primers managed by WASABI [Kauff et al., 2007].) Simultaneous with the rapid growth in the collection of multigene data sets was the dramatic development of algorithms for model-based phylogenetic analyses of complex nucleotide sequence datasets. Maximum likelihood and Bayesian analyses of large multigene data sets are now routine due to the development of faster and more powerful processors and readily available computer software packages with more efficient search algorithms (e.g., RAxML [Stamatakis, 2006]; GARLI [Zwickl, 2008]; MRBAYES [Ronquist and Huelsenbeck, 2003]; and PhyloBayes [Lartillot and Philippe, 2004]). Recent analyses of multigene sequenced data have resulted in enhanced phylogenetic resolution (Matheny et al., 2007; Hofstetter et al., 2007) and have demonstrated that the protein-coding genes *RPB1*, *RPB2*, and *TEF* possess higher levels of phylogenetic informativeness than rRNA genes (Townsend, 2007; Schoch et al., 2009). The convergence of our ability to collect relatively large amounts of multigene sequence data and our ability to rigorously analyze these data has resulted in our current and most accurate understanding of the evolution of the Fungi.

THE FUNGAL TREE OF LIFE

By the Fungal Tree of Life, we explicitly refer to the monophyletic Kingdom Fungi (Fungi) and all of its subgroups. For brevity's sake we will not discuss groups outside the Fungi (e.g., Oomycota), although they are important organisms at numerous levels and have traditionally been studied by mycologists. We also focus our discussion on higher-level systematics, emphasizing major clades of Fungi and patterns of fungal evolution. The Fungi currently comprises one subkingdom, six phyla, 10 subphyla, 35 classes, 12 subclasses, and 129 orders (Hibbett et al., 2007). These taxa collectively form a phylogenetic classification whereby all groups are based on phylogenetic analyses that revealed support for monophyletic groups (clades) of fungi (see Hibbett et al., 2007, for supporting studies). As such, some traditional groups were not inferred to be monophyletic and some taxa (e.g., Zygomycota) were not formally designated as a group, while others (e.g., Chytridiomycota) have a more restricted use. This classification will undoubtedly be modified over the years as new higher-level taxa are discovered, but the recognition of taxa based on the criterion of monophyly as revealed by explicit multigene phylogenetic analyses should provide a greater level of stability. Also, the names proposed for higher taxa by Hibbett et al. (2007) employ the rationale of autotypification as originally applied to the Ascomycota by Eriksson and Wynka (1997). That is, the names of higher taxa (e.g., class Sordariomycetes, subphylum Pucciniomycotina) are based on well-characterized and nomenclatural legitimate genera (e.g., *Sordaria* and *Puccinia*, respectively), thereby removing any taxonomic confusion or uncertainty associated with previous classifications (e.g., Pyrenomycetes and Urediniomycetes, respectively).

The Early-Diverging Lineages of the Fungal Tree of Life

The early-diverging lineages of the Fungal Tree of Life (Fig. 1) consist of zoosporic fungi formerly classified in the Chytridiomycota (James et al., 2006a), nonflagellated filamentous fungi of Zygomycota (White et al., 2006), and the Microsporidia (Keeling and Fast, 2002). Microsporidia are unicellular, intracellular parasites of animals, and their phylogenetic placement has been and continues to be controversial (Gill and Fast, 2006; Liu et al., 2006). They possess highly reduced genomes and relatively high rates of nucleotide substitution (Keeling and Slamovits, 2004). Consequently, there exist fewer clear-cut examples of shared orthologous genes with other taxa. For those genes that are clearly orthologous (e.g., rRNA genes), nucleotide positional homology is often difficult to assess due to the high rate of nucleotide substitution and significantly complicates DNA alignments and phylogenetic analyses. Historically Microsporidia were classified in the Protista, which has long been recognized as a taxon of convenience and not monophyletic. More recent phylogenetic analyses of protein-coding data support Microsporidia as a member of the early-diverging lineages of the Fungi (Keeling, 2003; James et al., 2006a) or as a sister group to the Fungi (Liu et al., 2006). Hibbett et al. (2007) classified Microsporidia as a member of the Fungi, which is consistent with either of the aforementioned placements. One of the more provocative hypotheses concerning the phylogenetic placement of Microsporidia is that of James et al. (2006b). In that study, which is based on five nuclear genes and one mitochondrial locus, Microsporidia, along with *Rozella*, a chytrid that is an intracellular parasite of other fungi, formed the earliest-diverging clade of the Fungi. Caution is advised here, however, as the grouping of these intracellular parasites may well be an artifact of long branch attraction driven by common molecular evolution processes (e.g., high rates of nucleotide substitution, reduced gene content, nucleotide biases, etc.). Consistent with this relationship being an analytical artifact, a more recent analysis of genome organization provided support for placement of Microsporidia among the zygomycetous fungi based on the presence and synteny of sex loci (Lee et al., 2008). Thus, the placement of both Microsporidia and *Rozella* remains elusive, and these two important taxa require additional research.

Although zoosporic (flagellated) fungi comprise a small number (<1,000) of known species of Fungi (Kirk et al., 2008), an accurate understanding of their phylogenetic relationships is essential to understanding early and pivotal events in the evolution of the kingdom. All zoosporic species of Fungi produce zoospores that possess a single smooth posterior whiplash flagellum with some polyflagellate exceptions. Until recently, zoosporic species of Fungi were classified in the Chytridiomycota sensu Barr (Barr, 1992). Phylogenetic analyses of multiple genes, however, have revealed that the Chytridiomycota does not form a monophyletic group (James et al., 2000, 2006a, 2006b). Rather, these fungi comprise a set of paraphyletic lineages that occupy much of the early-diverging branches of the Fungal Tree of Life (Fig. 1). These lineages include the genus *Rozella* (mentioned above), the Chytridiomycota, Neocallimastigomycota, and Blastocladiomycota, and the genus *Olpidium*. The Chytridiomycota represents the core chytrid clade, and most species produce zoosporangia with life cycles characterized by very limited if any hyphal growth. The majority of Chytridiomycota members are saprobic and occur in

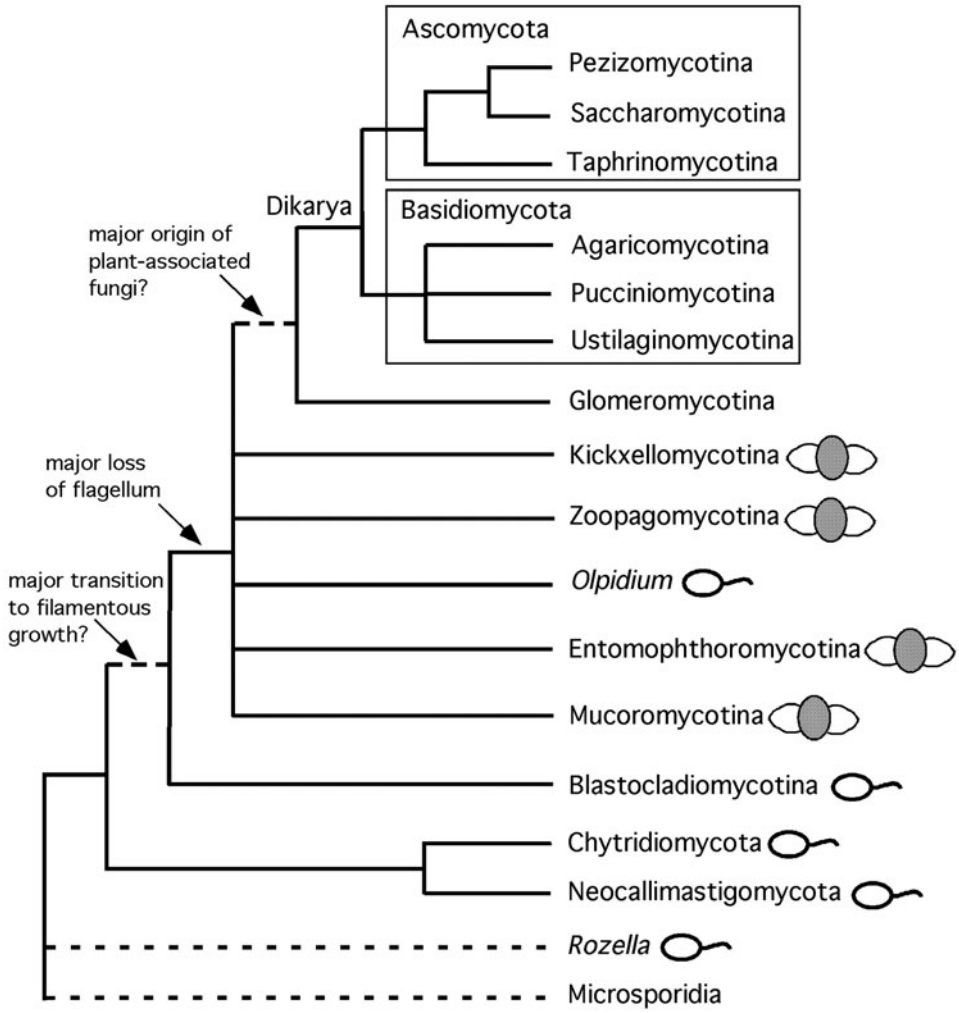


FIGURE 1 Fungal Tree of Life. Phylogenetic tree depicting the major phyla and subphyla of the Kingdom Fungi. Hypotheses for major morphological transitions are depicted along the backbone of the tree. Lineages of zoosporic and zygosporangium-producing fungi are shown along the right of the tree with the symbols of stylized zoospores and zygosporangia, respectively. Dashed lines represent early-diverging lineages of uncertain placement (e.g., *Rozella* and Microsporidia) or internal nodes that are resolved in cited studies but with <70% bootstrap values (e.g., Blastocladiomycota and Glomeromycota). Phylogenetic relationships are based on Lutzoni et al. (2004), James et al. (2006b), and Hibbett et al. (2007).

freshwater and terrestrial ecosystems, but a number of important pathogens do reside in this clade including the amphibian decline pathogen *Batrachochytrium dendrobatidis* and the potato pathogen *Synchytrium endobioticum*. It is a sister group to the Neocallimastigomycota, which includes fungi that produce polyflagellated zoospores. These fungi reside in the gastrointestinal system of ruminants, where they function in the breakdown of cellulosic material and are the only known example of obligate anaerobic fungi.

The Blastocladiomycota is the third major phylum of zoosporic fungi. These fungi produce copious amounts of filamentous hyphae and include examples of fungi with true alternations of generations (e.g., *Allomyces*). Other fungi may have an alternation of generations, e.g., *Saccharomyces cerevisiae*, but it is uncommon in Fungi. Current multigene phylogenies provide some support for the placement of Blastocladiomycota as a sister group to the remaining taxa of Fungi, but additional confirmation is needed (James et al., 2006a, 2006b). Finally, the genus *Olpidium*, a parasite of plant roots, is nested among the nonflagellated fungi traditionally classified in the Zygomycota and not with other "chytrids" (Fig. 1) (James et al., 2006b).

Our understanding of the phylogenetic relationships of the zoosporic fungi is central to one of the major questions concerning the Fungal Tree of Life, namely, the evolution of the flagellum. There is general consensus that the most recent common ancestor (MRCA) of Fungi possessed a flagellated stage in the life cycle, but there exist multiple hypotheses concerning the number of losses of the flagellum, which resulted in nonflagellated Fungi (e.g., Ascomycota and Basidiomycota). The work of Liu et al. (2006) supports a single loss of the flagellum during the evolution of the Fungi, whereas a study by James et al. (2006b), which sampled a larger number of taxa (e.g., *Rozella* and *Olpidium*)

and more genes, estimated multiple losses of the flagellum. Regardless, these rival hypotheses are consistent with a flagellated MRCA and the polarity of character state transition being ordered from flagellated to nonflagellated.

The remaining taxa of the early-diverging lineages of *Fungi* consist of species typically classified in the "Zygomycota" (Fig. 1). Most species grow as aseptate filaments, although septate hyphae are common in some groups (e.g., Trichomycetes of Kickxellomycotina), and they are characterized by gametangial copulation that results in the zygosporangium. Like the zoosporic fungi, zygosporangium-producing fungi do not form a monophyletic group. Rather, they form a paraphyletic grade, and the most accurate use of the term Zygomycota is currently under debate (White et al., 2006; Hibbett et al., 2007). For that reason the zygosporangium-producing fungi are classified in four monophyletic subphyla, Entomophthoromycotina, Kickxellomycotina, Mucoromycotina, and Zoopagomycotina, whose phylum level classification is *incertae sedis* (uncertain).

The Mucoromycotina comprises some of the best-studied species of zygosporangium-producing fungi including *Rhizopus* of the Mucorales, *Mortierella* (Mortierellales), and the mycorrhizal *Endogone* (Endogonales). As such, this clade may be thought of as the core clade of "Zygomycota." The remaining clades include the Entomophthoromycotina (Entomophthorales), a large group of insect pathogens, and the Zoopagomycotina (Zoopagales), which includes parasites and predators of amoebae, nematodes, and rotifers. Finally, the Kickxellomycotina includes an interesting assemblage of taxa that contains saprobes, mycoparasites (Kickxellales and Dimargaritales), and obligate symbionts of arthropod hindguts (Trichomycetes). While the zygosporangium-producing fungi do not form a monophyletic group, they do occupy a somewhat intermediate position between the zoosporic fungi and the Dikarya (Ascomycota plus Basidiomycota). rRNA data continue to dominate the currently available data for most zygosporangium-producing fungi (White et al., 2006), and a significant increase in protein-coding and genomic data is needed to resolve the evolutionary relationships of the zygomycete lineages and to develop a more complete understanding of the evolutionary origins of the nonflagellated terrestrial lineages of the Fungal Tree of Life.

Glomeromycota

The Glomeromycota represents one half of what is arguably the most successful symbiosis on the Earth. These fungi are the arbuscular mycorrhizae fungi that form endomycorrhizae with the majority of plant species on the planet (Smith and Reed, 2008). Previous evolutionary hypotheses considered these fungi to be members of the Endogonales (Mucoromycotina), but early rRNA phylogenies did not support this classification (reviewed by Redecker and Raab, 2006). Rather, these early studies suggested that the Glomeromycota (as Glomales) was a sister group to the Dikarya (Simon et al., 1993; Schüβler et al., 2001), resulting in the hypothesis that the MRCA to the Glomeromycota and Dikarya corresponded to the colonization of land by the green plant lineage. Consistent with this hypothesis are the numerous Rhynie Chert fossils of Glomeromycota associated with the earliest known land plants (Taylor et al., 2003) and that the majority of plant-associated fungi and mycorrhizae are members of the Glomeromycota plus Dikarya clade. The support for this relationship is tenuous, however, and multigene phylogenetic analyses have not contributed much to resolve this question (James et al., 2006b). Thus, we cannot discount the hypothesis that the Glomeromycota may not be a sister group to the Dikarya and that the plant associations and mycorrhizae characterized in the Glomeromycota and Dikarya may represent parallel origins of fungal-plant symbioses.

Dikarya

The subkingdom Dikarya comprises the majority of known fungal species (>95,000) and includes the phyla Ascomycota and Basidiomycota. It is diagnosed by the production of regularly septate hyphae and a dikaryotic nuclear state in the life cycle, although the extent to which it constitutes part of the life cycle varies greatly between the two phyla (Alexopoulos et al., 1986; Hibbett et al., 2007). Typically, the dikaryotic state is the long-lived, vegetative state in the Basidiomycota, but it is restricted to a short-lived ascogenous or sexual reproductive state in the Ascomycota. It is also among the Dikarya that multicellularity (fruit body production) occurs among the Fungi, likely as a product of independent evolutionary events within the Basidiomycota and Ascomycota.

The Basidiomycota comprises three subphyla: Pucciniomycotina, Ustilaginomycotina, and Agaricomycotina (Fig. 2). As previously mentioned, to avoid confusion between taxon names and informal names, each of these subphyla is named after a well-characterized genus (Hibbett et al., 2007). These subphyla, however, are ecologically and morphologically diverse and contain more than rusts, smuts, and mushrooms, respectively. The relationships of the three subphyla to one another are essentially unresolved, with the backbone of the Basidiomycota currently depicted as a trichotomy of these three taxa.

The Pucciniomycotina is the second largest subphylum of Basidiomycota and includes eight classes and 18 orders. While most researchers in fungal biology are familiar with the rust fungi (*Puccinia*), this subphylum also includes basidiomycetous yeasts (Sporidiobolales), smut fungi (Microbotryales), insect symbionts (Septobasidiales), and fungi formerly classified in Ascomycota (Mixiales) (Aime et al., 2006). Thus, the Pucciniomycetes is one of the more diverse clades of fungi and remains one of the more challenging experimental groups due to their often biotrophic nature and complex life histories.

The Ustilaginomycotina includes two classes and nine orders and the majority of plant pathogenic "smut fungi" (Begerow et al., 2006). It is in the Pucciniomycotina and Ustilaginomycotina that we see the production of teliospores, specialized spores in which karyogamy and meiosis occur and on which promycelia (modified basidia) are produced.

The Agaricomycotina comprises three classes (Tremellomycetes, Dacrymycetes, and Agaricomycetes) and 21 orders of fleshy, sporocarp-forming Basidiomycota (Hibbett, 2006). The Tremellomycetes is the earliest-diverging lineage of the Agaricomycotina and includes jelly fungi and species with both filamentous and yeast growth forms. These fungi include agents of wood decay, mycoparasites, and important human pathogens of increasing medical importance (e.g., *Cryptococcus* spp.). The Dacrymycetes also produce gelatinous sporocarps that tend to fruit from woody substrates, but it shares a more recent common ancestor with the Agaricomycetes than with the Tremellomycetes. The Agaricomycetes includes all of the fleshy basidiomycetes that are typically referred to as mushrooms, boletes, conchs, crusts, and puffballs. It represents the largest group of known Basidiomycota and arguably the most important group of fungi associated with forest ecosystems, including ectomycorrhizae, tree pathogens, and wood and litter decay. It also includes most of the species commonly

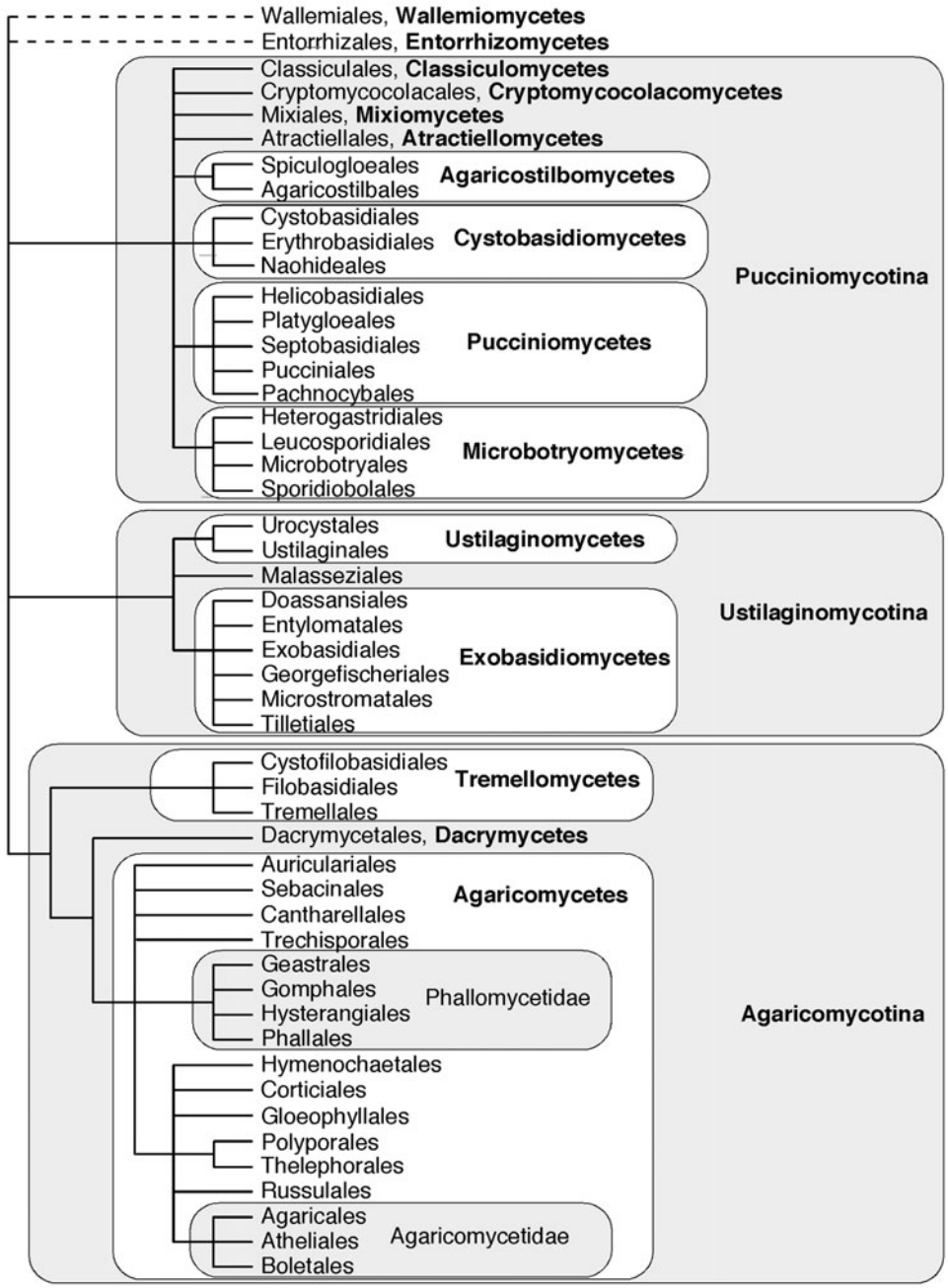


FIGURE 2 Phylogeny and classification of Fungi: Basidiomycota. Dashed lines indicate taxa that are of uncertain placement. Reproduced with permission from Hibbett et al., 2007.

cultivated in the commercial mushroom industry (e.g., Chinese black jelly mushroom [*Auricularia* spp.], button mushroom [*Agaricus bisporus*], shiitake mushroom [*Lentinula edodes*], oyster mushroom [*Pleurotus ostreatus*], etc.) and some of the more prized culinary mushrooms (e.g., chanterelles [*Cantharellus* spp.], porcini [*Boletus edulis*], and matsutake [*Tricholoma* spp.]). Some of the more characterized model organisms in terms of basidiomycete genomics, genetics, and cell biology (e.g., *Coprinopsis cinereus*) are also members of the Agaricomycetes.

Considerable advancements have been made in the understanding of morphological and ecological character evolution of the Basidiomycota as a function of multigene phylogenies. Septate basidia and repetitive spore germination are present in the Pucciniomycotina, Ustilaginomycotina, Tremellomycetes, Dacrymycetes, and Agaricomycetes and are likely ancestral for the Basidiomycota, with traditional (agaricoid) nonseptate holobasidia and basidiospore germination restricted to germ tube formation primarily confined to the Agaricomycetes (Hibbett, 2006). A saprobic ecology (e.g., wood and litter decay) is ancestral for most lineages with multiple independent derivations of ectomycorrhizae (James et al., 2006b; Matheny et al., 2006; Binder and Hibbett, 2006; Hibbett and Matheny, 2009). Finally, sporocarp morphology is a poor predictor of evolutionary relationships. This is perhaps the most dramatic impact of molecu-

lar phylogenetics, as many of the preexisting hypotheses regarding evolutionary relationships and their classifications were based on the sporocarp morphology (Hibbett and Binder, 2002; Matheny et al., 2006, 2007). Ancestral character state reconstructions of sporocarp morphology on robust molecular phylogenies have revealed that sporocarp morphologies are some of the more labile traits in fungal evolution (Hibbett, 2004). It appears likely that all major sporocarp morphologies (e.g., mushrooms, puffballs, conches, crusts, etc.) have been derived multiple times during the evolution of the Basidiomycota. Currently there are 11 major ordinal and/or supraordinal clades recognized within the Agaricomycetes, the majority of which include multiple sporocarp morphologies. (See Matheny et al., 2007, on Agaricomycetes systematics.)

Ascomycota

With more than 63,000 species, the Ascomycota is the largest phylum of Fungi (Kirk et al., 2008) and like the Basidiomycota includes three subphyla: Taphrinomycotina, Saccharomycotina, and Pezizomycotina (Fig. 3). Taphrinomycotina (~140 spp.) represents the earliest-diverging lineage of the Ascomycota and is a sister group to the Saccharomycotina plus Pezizomycotina clade. It includes four classes of morphologically and ecologically distinct fungi including plant pathogens (*Taphrina*, Taphrinomycetes), human pathogens (*Pneumocystis*, Pneumocystidiomycetes), fission yeasts (*Schizosaccharomyces*, Schizosaccharomycetes) and sporocarp-producing taxa (*Neolecta*, Neolectomycetes). Its monophyly has been debated for some time with early rRNA analyses resolving the group as a paraphyletic grade at the base of the Ascomycota, while other multigene analyses have supported its monophyly (Sugiyama et al., 2006). The most recent analyses of six genes and increased taxon sampling have provided additional support for its monophyly (James et al., 2006b; Spatafora et al., 2006), and it is recognized as a formal subphylum (Hibbett et al., 2007).

The Saccharomycotina are the "true yeasts" and include the model organism *S. cerevisiae* and important human pathogens such as *Candida albicans*. Few fungi have had a more pronounced impact on human civilization than *S. cerevisiae* due to its use in fermentation and the production of alcohols and leavened breads. While there is a single order (Saccharomycetales) and relatively few described species (~1,000 [Kirk et al., 2008]), environmental sampling of novel ecological niches such as beetle endosymbionts has revealed a staggering diversity of Saccharomycotina yeasts (Suh et al., 2005). This research has demonstrated that many tropical species of wood and fungus-inhabiting beetles possess obligate fungal endosymbionts that are members of the Saccharomycotina. The exact function of most of these fungi is unknown, but extrapolations from other fungal endosymbionts of insects suggest that they may play a role in fatty acid metabolism and/or the breakdown of noxious compounds. If this rate of species discovery continues, it is not unreasonable to assume that the Saccharomycotina may be one of the most species-rich clades of Fungi.

The Pezizomycotina are what most researchers in fungal biology refer to as filamentous fungi or filamentous ascomycetes, although it does contain a few species with yeast-like growth (e.g., *Symbiotaphrina*). It includes 10 classes (Fig. 3), 55 orders, and more than 60,000 species (Kirk et al., 2008). Importantly, classes of the Pezizomycotina include some of the better-studied model organisms such as *Neurospora* of the Sordariomycetes and *Aspergillus* and *Penicillium* of Eurotiomycetes. The relationships among the 10 classes of Pezizomycotina remain one of the more recalcitrant problems in fungal phylogenetics, but some patterns are emerging and statistically well-supported relationships do exist (Spatafora et al., 2006; Schoch et al., 2009). The earliest-diverging lineages of the phylum are Orbiliomycetes (e.g., *Orbilia*; nematode trappers and saprobes) and Pezizomycetes (e.g., *Ascobolus* and *Peziza*; saprobes and ectomycorrhizae), two classes of fungi that form cup-shaped sporocarps (apothecia).

The remaining classes form a monophyletic supraclass level clade, "Leotiomyceta," which is characterized by a basal polytomy and considerable morphological and ecological diversity (Lumbsch et al., 2005; Schoch et al., in press). Within the "Leotiomyceta," the Sordariomycetes (e.g., *Neurospora*) and Leotiomycetes (e.g., *Sclerotinia*) form a monophyletic group that primarily includes perithecial (flask-shaped) and apothecial fungi, respectively, but is united by the production of relatively thin-walled, unitunicate asci (Spatafora et al., 2006; Robbertse et al., 2006; Schoch et al., 2009). The Dothideomycetes (e.g., *Pyrenophora*) and Arthoniomycetes (e.g., *Arthonia*) form a monophyletic group that includes plant saprobes/pathogens and lichens, respectively, but is united by the production of thick-walled bitunicate asci (Schoch et al., 2006). Additional major classes of the "Leotiomyceta" include the Eurotiomycetes and Lecanoromycetes. The Eurotiomycetes (e.g., *Aspergillus* and *Penicillium*) represents one of the most morphologically and ecologically diverse classes of fungi, comprising saprobes, human pathogens, plant pathogens, mycorrhizae, lichens, and rock-inhabiting fungi. Its monophyly has been debated with analyses and arguments both in favor of (Lutzoni et al., 2004) and against (Liu and Hall, 2004) its monophyly and recognition as a class. More recent multigene analyses, however, have supported its monophyly and provide an excellent example of where increased taxon and character sampling confidently resolved a complex question in fungal phylogenetics (Geiser et al., 2006). Finally, the Lecanoromycetes (e.g., *Lecanora* and *Peltigera*) contains the vast majority of lichenized fungi. While a core set of families and orders are confidently placed in the class, the boundaries of the class are debatable, as are its relationships to the other classes of the "Leotiomyceta" (Miadlikowska et al., 2006; Hofstetter et al., 2007).

Like the Basidiomycota, molecular phylogenetics has provided increased insight into the ecological and morphological evolution of the Ascomycota. A saprobic lifestyle is supported as ancestral for the phylum, with multiple derivations of mycorrhizae, plant pathogens, animal pathogens, and lichens (James et al., 2006b; Schoch et al., in press). More than 98% of all known lichens are ascomycetes, and they account for approximately 25% of Pezizomycotina species diversity (based on estimated species numbers for Arthoniomycetes [~1,600] and Lecanoromycetes [~14,000] [Kirk et al., 2008]). The exact number of lichenization events is not fully resolved (Lutzoni et al., 2001; Schoch et al., in press), but increased taxon and character sampling supports all ascomycete lichens being restricted to the Pezizomycotina and at least two origins of lichenization within the subphylum (Lecanoromycetes and Arthoniomycetes). Prior to molecular phylogenetics, many of the evolutionary hypotheses and the resulting classifications of the Ascomycetes were based on sporocarp (e.g., apothecia, perithecia, cleistothecia, and pseudothecia), ascus (e.g., operulate, inoperulate, bitunicate, and unitunicate), and developmental (e.g., ascohymenial and ascolocular) characters (reviewed by

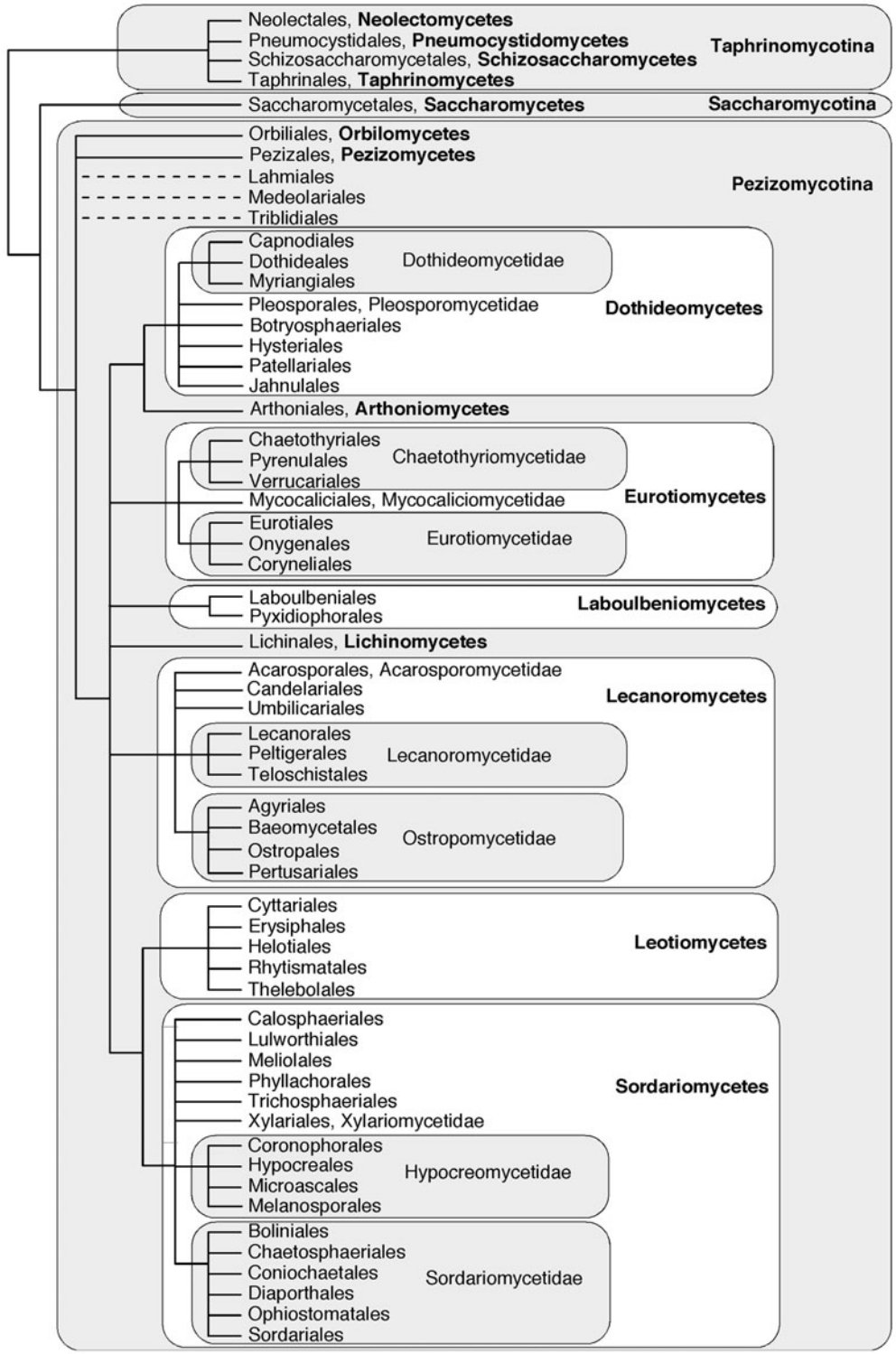


FIGURE 3 Phylogeny and classification of Fungi: Ascomycota. Dashed lines indicate taxa that are of uncertain placement. Reproduced with permission from Hibbett et al., 2007.

Spatafora et al., 2006). Most of these characters are not diagnostic of natural, monophyletic groups, and the classic names used to designate formal taxa (Discomycetes for apothecia, Pyrenomycetes for perithecia, Plectomycetes for cleistothecia, and Loculoascomycetes for pseudothecia) have been abandoned (Hibbett et al., 2007) and now refer to morphologies only. These characters either are ancestral and have been lost multiple times (e.g., the apothecium) or derived multiple times during the evolution of the Ascomycota (e.g., the cleistothecium), or are not diagnostic of a monophyletic group with equivocal explanations of character state gains and losses (e.g., bitunicate asci of

Eurotiomycetes and Dothideomycetes/Arthoniomycetes) (Schoch et al., in press). The names used in the formal higher classification of the Ascomycota are not, therefore, meant to impart morphologies but are clade designators based on resident genera (e.g., Sordariomycetes [*Sordaria*], Eurotiomycetes [*Eurotia*], etc.).

DATING THE FUNGAL TREE OF LIFE

One of the more elusive areas of research in fungal phylogenetics has been the calibration of the Fungal Tree of Life to geologic time (Taylor and Berbee, 2006). As we increase our understanding of the major groups of Fungi and their evolutionary relationships, we would like to place the Fungal Tree of Life into the context of the evolution of the Earth and its ecosystems and the origin and diversification of other major life forms (e.g., plants and animals). Part of the problem, however, is the paucity of fungal fossils that can be confidently assigned to a given lineage. Exceptions do exist and include >400 million-year-old Glomeromycota fossils (Taylor et al., 2003; Redecker et al., 2000), ~90 million-year-old mushrooms of the Agaricales (Hibbett et al., 1995), and ~100 million-year-old insect pathogens of the Hypocreales (Sung et al., 2008). The second major problem is that most data sets do not behave in a strictly clock-like fashion, and while advancements have been made to accommodate relaxed molecular clocks, no one methodology is universally accepted (e.g., r8s [Sanderson, 2003], PhyloBayes [Lartillot and Philippe, 2004], and BEAST [Drummond and Rambaut, 2007]).

The collective result is a very broad spectrum of dates for the last common ancestor of Animals and Fungi that has been estimated to be anywhere from 2.5 billion years old to 600 million years old depending on the calibration point(s) used (Heckman et al., 2001; Hedges and Kumar, 2004; Taylor and Berbee, 2006) (Fig. 4). Because most fungal fossils are found either associated with fossil plants or in terrestrial paleoecosystems with well-developed floras, it seems reasonable to postulate that the diversification of most extant lineages of Fungi is closely associated with the origin and diversification of land plants and terrestrial ecosystems (Taylor and Berbee, 2006).

PHYLOGENOMICS OF THE FUNGAL TREE OF LIFE

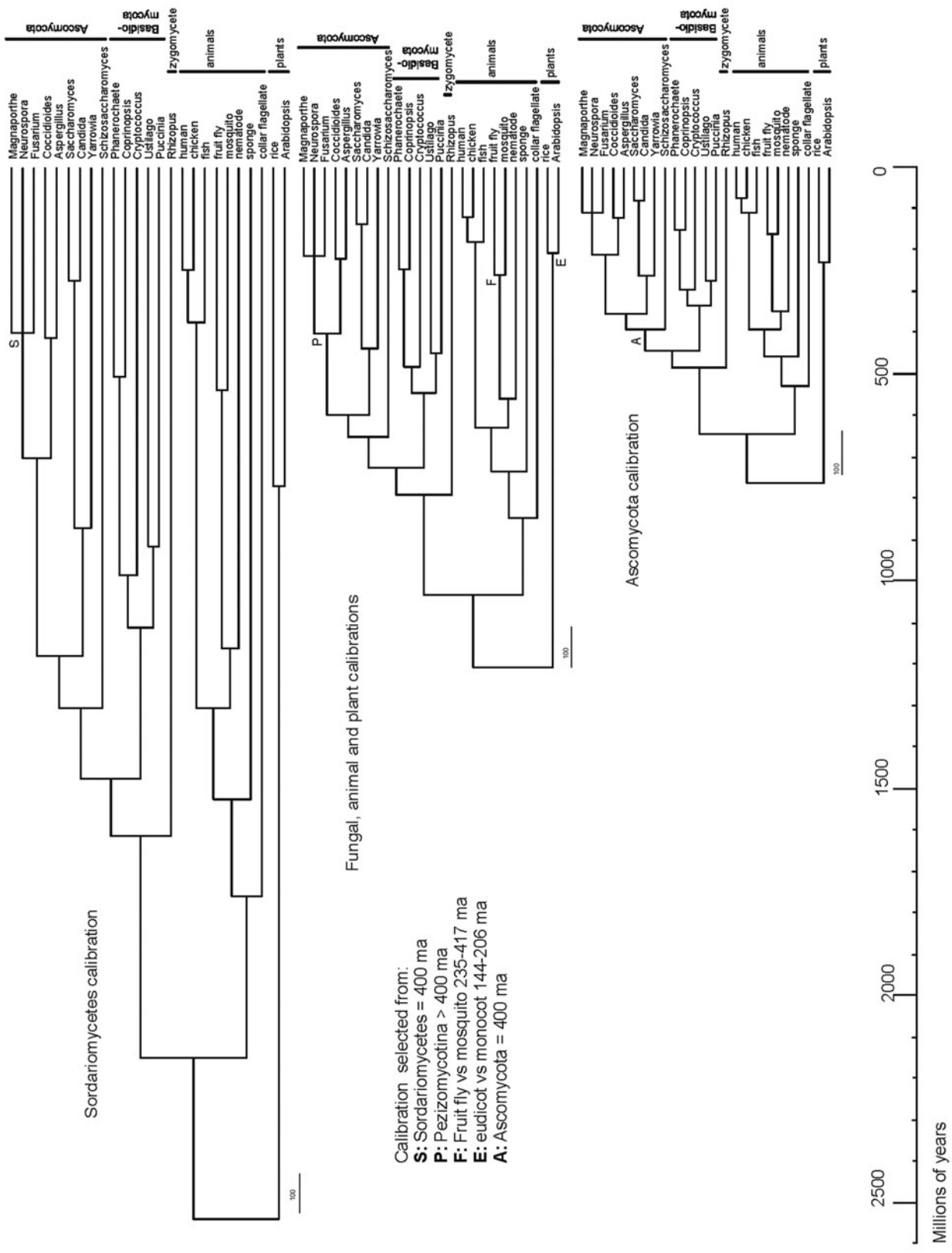
As stated previously, phylogenomics is a term used broadly with several definitions (Philippe et al., 2005; Delsuc et al., 2006). Here we focus on the use of genome-scale data sets in phylogenetic analyses. Currently there have been more genomes sequenced for Fungi than for any other kingdom of eukaryotes, and at the preparation of this text more than 100 genomes in different stages of annotation were publicly available (http://www.genomesonline.org/ and http://fungalgenomes.org/genome). The majority of these have been species of Ascomycota, with a more recent increase in the sampling of Basidiomycota with a particular emphasis on wood decay and plant and human pathogens. Many clades of Fungi are lacking sequence information for exemplar genomes, especially early-diverging lineages (e.g., zoospore and zygosporangium-producing fungi). This situation poses a significant problem with respect to taxon sampling and the error that undersampling can introduce into phylogenetic analyses (Baurain et al., 2007). This problem will be alleviated in due course, however, as genomic sequencing becomes less expensive and more widely available to basic fungal biology laboratories. Below we provide a brief review of some of the analytical issues such as ortholog discovery and inferring nodal support, and then we provide a brief review of published phylogenomic analyses of fungi.

ORTHOLOG DISCOVERY AND NODAL SUPPORT

Molecular phylogenetics is dependent on the analysis of orthologous genes, with the misinterpretation of paralogous copies leading to erroneous estimation of species trees from gene trees. The problem of paralogy is greatly exacerbated in genomic data, where many if not most of the genes are initially unknown and they are part of gene families characterized by complex patterns of gene loss and gene duplication. Furthermore, the assessment of orthology relies on well-annotated genomes, so any error intrinsic to a given genome annotation will be introduced into the initial stages of analysis and perpetuated. In an attempt to begin to unravel these complex patterns of homology, different algorithms and methodologies have been developed. Most methods and algorithms for estimating homology and orthologous groups of proteins have been applied to the annotations of genomes, not necessarily for the selection of phylogenetic markers.

Recently, Kuzniar et al. (2008) provided a comprehensive review of published programs and databases of ortholog identification in which the strengths and weaknesses of each are highlighted. Some of the major detection methods are informed by precompiled ortholog databases (COCO-CL [Jothi et al., 2006]; Orthostrapper [Storm and Sonnhammer, 2002]; LOFT [van der Heijden et al., 2007]; RIO [Zmasek and Eddy, 2002]), while others are ab initio programs, such as Inparanoid (Remm et al., 2001), Ortholuge (Fulton et al., 2006), MultiParanoid (Alexeyenko et al., 2006), and OrthoMLC (Li et al., 2003). Of the ab initio programs only the last two are capable of making ortholog predictions across multiple genomes, an attractive quality as phylogenetic analyses are inherently comparisons of multiple taxa. Furthermore, Markov clustering methodologies (van Dongen, 2000) are more applicable to automation than KOG (Tatusov et al., 2003), which relies on manual curation. These Markov clustering methods, however, make tradeoffs between sensitivity and specificity, resulting in different levels of false-positive and false-negative error rates (Chen et al., 2007). While these rates can be estimated with well-characterized genomes with high confidence in gene models and subsequent annotations, their application to the incorporation of newly sequenced genomes into phylogenetic analyses will likely involve examples of both false positives (inclusion of nonorthologous proteins) and false negatives (exclusion of orthologous proteins). Undoubtedly this field of research will undergo considerable maturation over the next several years.

Two important revelations that occurred early in the development of phylogenomic analyses were observable conflicts among individual gene trees and the difficulty in assessment of nodal support, or the statistical confidence, in a particular clade. Due to processes such as gene duplication, gene conversion, lineage sorting, and horizontal gene transfer, different genes can have evolutionary histories that are in conflict with one another and that do not accurately record the evolutionary history of the species. As genome-scale data are analyzed, it is inevitable that larger suites of genes with conflicting gene histories will be detected (Rokas et al., 2003). This pattern of conflict will likely become more apparent and will require informed choices of single-copy orthologous genes (Robbertse et al., 2006), application of accurate DNA and amino acid models of evo-


Ascomycota
Basidio-mycota
Zygomycete
animals
plants
Magnaporthe
Neurospora
Fusarium
Coccidioides
Aspergillus
Saccharomyces
Candida
Yarrowia
Schizosaccharomyces
Phanerochaete
Coprinopsis
Cryptococcus
Ustilago
Puccinia
Rhizopus
human
chicken
fish
fruit fly
mosquito
nematode
sponge
collar flagellate
rice
Arabidopsis
S
P
F
E
A
100
Sordariomycetes calibration
Fungal, animal and plant calibrations
Ascomycota calibration
Calibration selected from:
S: Sordariomycetes = 400 ma
P: Pezizomycotina > 400 ma
F: Fruit fly vs mosquito 235-417 ma
E: eudicot vs monocot 144-206 ma
A: Ascomycota = 400 ma
2500
2000
1500
1000
500
0
Millions of years

lution (Philippe et al., 2005; Liu et al., 2009), and scaleable tests for phylogenetic conflict (Kauff and Lutzoni, 2002).

In traditional phylogenetic analyses the most common measure of nodal support is the nonparametric bootstrap (Felsenstein, 1985), or bootstrap proportions (BPs), and the posterior probability (Ronquist and Huelsenbeck, 2003). The latter is generally considered to be a liberal statistic, and more emphasis is placed on the BP (Suzuki et al., 2002; Alfaro et al., 2003; Taylor and Piel, 2004). Importantly, BPs are not confidence intervals, and a node is typically considered well supported when it receives greater than 70 to 75% BP (Lutzoni et al., 2004). When applied to phylogenomic analyses, however, BPs are rarely below 70 to 75%, even though some of the nodes are unstable, i.e., in conflict across different data sets, and sensitive to taxon and character sampling (Rokas et al., 2003; Philippe et al., 2004, 2005; Robbertse et al., 2006; Fitzpatrick et al., 2006). This is particularly troubling as different methods of ortholog detection result in different sets of putative orthologous proteins (Chen et al., 2007), and, as previously mentioned, phylogenomic analyses in general suffer from inadequate taxon sampling. This situation is currently unresolved, and BPs should be viewed conservatively with any knowledge of how different taxon samplings, character samplings, and differences in analyses (e.g., characters coded as DNA versus amino acid) affect the BP of a given node.

GENOME-SCALE PHYLOGENETIC ANALYSES OF FUNGI

The first genome-scale phylogenetic analyses that included fungal sequences were performed by Rokas et al. (2003). This study analyzed 106 genes from eight taxa in the Saccharomycotina. The 106 individual gene trees resulted in more than 20 alternative topologies, whereas analyses of a concatenated alignment of all 106 genes produced a single well-supported topology that was inconsistent with the 20 alternative single-gene topologies in at least one inference. Jeffroy et al. (2006) analyzed the same 106 genes in the Rokas et al. (2003) study but expanded the taxon sampling to 14 Saccharomycetina species. Their analyses explained the incongruence among the single-gene trees as a result of compositional biases, saturation, and method of analysis. The latter analysis also provided strong support for the WGD (whole-genome duplication) and CTG (organisms translate CTG as serine instead of leucine) clades within Saccharomycotina.

Robbertse et al. (2006) produced the first study of a wider sampling of the Ascomycota. Their analyses included a concatenated alignment of 781 genes from 17 taxa (the Saccharomycotina [4 taxa], Pezizomycotina [10 taxa], Schizosaccharomycotina [1 taxon], and 2 Basidiomycota taxa as outgroup). Neighbor-joining and maximum likelihood analyses resulted in identical topologies, whereas the maximum parsimony analyses resolved the *Staganospora nodorum* (the only taxon representing the Dothideomycetes) differently. Uncertainty in the placement of *S. nodorum* was also found by Fitzpatrick et al. (2006) (Fig. 5); however, all higher taxa (e.g., classes) in which more than one taxon was sampled (e.g., Sordariomycetes, Eurotiomycetes, Leotiomycetes, etc.) were confidently resolved. Importantly, Robbertse et al. (2006) demonstrated the efficacy of analyses that combined ortholog discovery, protein alignments, and phylogenetic inference in a semi-automated computational pipeline. Expanding the sampling of fungal taxa, Fitzpatrick et al. (2006) inferred relationships between 42 species of Fungi (Pezizomycotina [17 species], Saccharomycotina [19 species], Taphrinamycotina [1 species], Basidiomycotina [4 species], and Zygomycotina [1 species]) using both supertrees analyses of 4,805 single-gene trees and analysis of a superalignment of 153 universally distributed orthologs. The consensus supertree reflected some artifacts, but a matrix representation with parsimony supertree and the phylogeny inferred from the concatenated alignment of 153 genes (Fig. 5) were highly congruent. The study identified two classes within the CTG clade of the Saccharomycotina that may correlate with sexual status. These analyses and others (Robbertse et al., 2006; Kuramae et al., 2006; Liu et al., 2009) supported the Leotiomycetes-Sordariomycetes sister group relationship to the exclusion of the remaining classes of the Pezizomycotina, resolving the homology of their unitunicate, inoperculate (poricidal) asci and demonstrating the plasticity of sporocarp morphology at the superclass level (Schoch et al., 2009).

In the first large-scale comparison of nuclear and mitochondrial genomic data, Liu et al. (2009) conducted a phylogenomic analysis using 113 nuclear genes and 13 mitochondrial genes involving taxa from which whole-genome or expressed sequence tag (EST) sequences were available. The sampling included taxa from the Pezizomycotina (16 taxa), Saccharomycotina (13 taxa), Taphrinamycotina (4 taxa), Basidiomycotina (10 taxa), Zygomycota (6 taxa), and Chytridiomycota (5 taxa) with the goal of testing the monophyly of the Taphrinomycotina. As a result of using EST data, the 113 nuclear gene data set had a proportion of missing data, with only one single protein represented for all 54 species and the average proportion of missing data being 25% per species. The authors concluded that the missing data did not affect the phylogenetic analysis significantly when using a large data set with a strong phylogenetic signal, but they did detect conflict between the nuclear and

FIGURE 4 Different calibration points change estimated divergence dates. Ascomycota split from Basidiomycota after the origin of two phyla not shown, the Chytridiomycota and Glomeromycota. In the top diagram we assumed that the 400 million-year-old fossil *Paleopyrenomycites devonicus* represents Sordariomycetes as indicated by the letter "S." This pushes the minimum age for the origin of the stem lineages of all five fungal phyla, including the Glomeromycota, to 1,489 million years ago, more than three times the age of the first fossil evidence of land plants. In the middle diagram we assumed that *P. devonicus* represents Pezizomycotina but not necessarily Sordariomycetes. Applying calibrations from plant and animal fossils in addition to assuming that *P. devonicus* represents Pezizomycotina gave an estimate of 792 million years ago for the origin of the fungal phyla, still almost twice the age of the first fossil evidence for vascular plants. In the bottom diagram, assuming that *P. devonicus* provided a minimum age for the Ascomycota, indicated by the letter "A," at 400 million years resulted in the estimate that fungal phyla had been established by 452 million years ago, roughly the age of the first land plants. In this scenario, however, the ages for the divergences among vertebrates are far too recent given fossil data. This discrepancy might result from using fossils from one kingdom (Fungi) to calibrate events in the same kingdom and another kingdom (Animalia), as discussed in the text. Reproduced with permission from Taylor and Berbee, 2006.

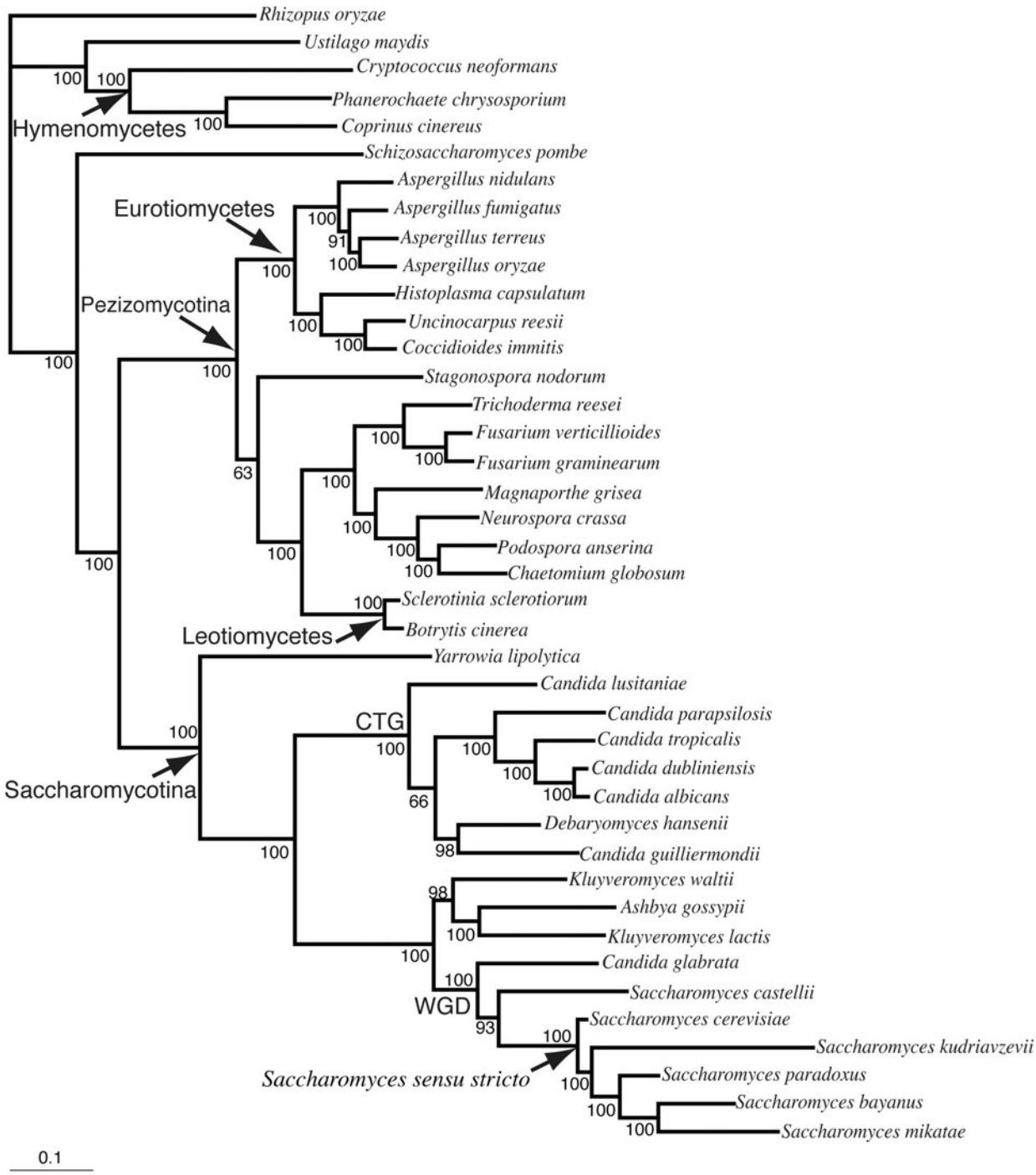


FIGURE 5 Maximum likelihood phylogeny reconstructed using a concatenated alignment of 153 universally distributed fungal genes. The concatenated alignment contains 42 taxa and exactly 38,000 amino acid positions. The optimum model according to ModelGenerator (Keane et al., 2006) was found to be WAG+I+G. The number of rate categories was 4 (alpha 5 0.83), and the proportion of invariable sites was approximated at 0.20. Bootstrap scores for all nodes are displayed. *Saccharomyces castellii* is found at the base of the WGD node. Reproduced with permission from Fitzpatrick et al., 2006.

mitochondrial data sets. The mitochondrial data of yeasts (Saccharomycotina) and fission yeasts (*Schizosaccharomyces*, Taphrinomycotina) were characterized by fast-evolving sequences, which resulted in patterns consistent with long-branch attraction. After they accommodated for fast-evolving positions, their analyses supported the Taphrinomycotina (using the following taxa as representatives: *Pneumocystis carinii*, *Saitoella complicata*, *Schizosaccharomyces pombe*, and *Taphrina deformans*) as monophyletic and as a sister group to the Saccharomycotina plus Pezizomycotina clade.

CONCLUSION

Multigene analyses have had a major impact on phylogenetic studies of the Fungi, resulting in our most thorough understanding of evolutionary relationships of the Kingdom to date (Blackwell et al., 2006; Hibbett et al., 2007). The advent of whole-genome sequencing and the inclusion of heterogeneous genomic sequence data (e.g., ESTs) has set the stage for addressing phylogenetic questions with the maximum amount of discrete, orthologous sequence data. Although the genome era provides an abundance of genes and sequence data, significant challenges remain in the assessment of orthology (Kuzniar et al., 2008) and in the analyses of problematic sequence data (e.g., saturated sites and fast-evolving positions [Jeffroy et al., 2006; Liu et al., 2009]) and heterogeneous supermatrices (Philippe et al., 2004; Delsuc et al., 2006).

Taxon sampling is currently the major limiting factor in genome-scale phylogenetic analyses, and attention and priority should be given to major clades of Fungi for which no genome data are available (e.g., Lecanoromycetes and Dacrymycetes). Finally, the application of new high-throughput sequencing technologies (e.g., Illumina, 454, etc.) to small eukaryotic genomes and cDNA libraries, while advancing broader taxon sampling of the Fungi, will diversify the types of sequence data available for phylogenetic analyses and likely require new analytical approaches.

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5

Signal Transduction Pathways

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Most filamentous fungi spend at least a portion of their life cycle as free-living organisms and, as such, are exquisitely sensitive to changes in their environment. Furthermore, those fungi that can parasitize or live symbiotically with another organism must sense the presence of the host in order to ensure a productive association. Such sensing and integration of signals from multiple sources—from both the abiotic and biotic environment—require a complex web of signal transduction pathways.

This chapter covers major signal transduction pathways that have been characterized in multiple species of filamentous fungi. Since many of these systems are elaborated upon in subsequent chapters in this book, the coverage is broad and summarizes recurring themes. The signaling pathways included are monomeric and heterotrimeric GTP binding proteins, mitogen-activated protein kinases (MAPKs), protein kinase A/cyclic AMP (PKA/cAMP) signaling, two-component regulatory systems, calcium signaling, target of rapamycin (Tor) pathways and pH regulatory mechanisms. With the exception of two-component systems, related pathways are found in animals, where they also play fundamental roles. In general, the elements of these systems are found in all fungal species that have been sequenced; however, the number of genes representing each signaling protein class often varies. In spite of this conservation, several interesting variations in how pathway components are arranged or regulated have also emerged. Furthermore, accumulating evidence indicates that multiple pathways often cooperate to regulate the same function in the same species, resulting in more complex signal transduction networks.

GTP-BINDING PROTEINS

Monomeric GTPases

Monomeric GTP-binding proteins, included in the superfamily of Ras-related small GTPases, participate in various cellular processes in eukaryotes (for reviews, see Jaffe and Hall, 2005, and Weeks and Spiegelman, 2003). Small GTPases cycle between GDP-bound inactive and GTP-bound active forms. The switch from the GDP- to GTP-bound form upon signal sensing is mediated by a guanine nucleotide exchange factor (GEF) that is specific to the particular GTPase. Conversion back to the inactive GDP-bound form is facilitated by a GTPase-activating protein (GAP). Another regulatory factor, the guanine nucleotide dissociation inhibitor (GDI), inhibits dissociation of GDP from the GTPase and membrane binding in order to prevent autoactivation.

Within the Ras superfamily, Ras and Rho subfamily proteins have been the most frequently implicated in the regulation of signal transduction in yeasts and filamentous fungi. Most filamentous fungi have two Ras proteins (Ras1p and Ras2p), but only a single Ras protein (RasAp) has been identified in *Aspergillus nidulans* (Som and Kolaparthi, 1994; Weeks and Spiegelman, 2003). Functional analysis of Ras proteins in these filamentous fungi demonstrates that they participate in the regulation of conidiation, mating, and pathogenesis by mediating signal transduction either from a Gα subunit to a downstream cAMP-dependent PKA pathway or from a pheromone receptor to an MAPK cascade. In *Neurospora crassa*, a homologue of mammalian Rap (another member of the Ras subfamily) is implicated in mating (Ito et al., 1997).

Among Rho subfamily GTPases, Rho, Cdc42, and Rac-related proteins are universally present in filamentous fungi. These GTPases regulate polarized growth of fungal hyphae, multicellular development and pathogenicity through cytoskeleton reorganization and other regulatory functions (Sudbery, 2008). Homologues of *Saccharomyces cerevisiae* Rho1p, Rho3p, and Rho4p have been identified in several filamentous fungi and characterized for roles in cell integrity, septation, polarized hyphal growth, and pathogenicity (Martinez-Rocha et al., 2008; Rasmussen and Glass, 2005; Wendland and Philippsen, 2001). Although Cdc42 is highly conserved among yeasts and filamentous fungi, Rac is found in filamentous fungi but absent from yeasts. Results from studies in several filamentous fungi show that Cdc42 and Rac GTPases possess distinct and common essential functions during regulation of polarized growth and morphological development (Momany, 2005; Sudbery, 2008). Thus, deletion of genes for both

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GTPases is lethal in fungi. In addition, accumulating evidence indicates that Cdc42 and Rac proteins can be regulated by Ras and then go on to activate downstream effectors, such as p21-activated kinases, in filamentous fungi (Chen et al., 2006; Vallim et al., 2005).

GEFs, GAPs, and GDIs control the activation state of their partner GTPase. The number of GEF and GAP genes found in the genomes of filamentous fungi is greater than that of small GTPases, suggesting the possibility of fine-tuning in the control of small GTPases (Borkovich et al., 2004). GAPs that inhibit the function of Rho1 have been identified in *Ashbya gossypii* and *N. crassa* (Vogt and Seiler, 2008; Wendland and Philippsen, 2000). Cdc24 and Cdc25-like proteins have been characterized as GEFs specific for Cdc42 and Ras-type GTPases, respectively, in several filamentous fungi (Muller et al., 2003; Nichols et al., 2007; Schink and Bolker, 2008). Most GEFs specific for Rho, Cdc42, and Rac-type GTPases in mammalian systems are distinguished by the presence of functional domains, such as the catalytic dbl homology, lipid-binding pleckstrin homology, and the zinc finger Fab1-YOTB/ZK632.12-Vac1-EEA1 domains, which are also found in GEFs from filamentous fungi (Rossman et al., 2005; Schink and Bolker, 2008). Functional characterization of these domains will reveal those that are important for temporal and spatial regulation of GEFs for small G proteins in filamentous fungi.

Heterotrimeric G Proteins

Heterotrimeric G proteins regulate numerous cellular processes during development and pathogenesis in filamentous fungi (for a review, see Li et al., 2007). Heterotrimeric G proteins, composed of α, β, and γ subunits, have universal roles as mediators, transferring signals from G-protein-coupled receptors (GPCRs) to downstream effectors in eukaryotes. In the inactive state, the complex of three subunits, with the Gα subunit in the GDP-bound form, is associated with a GPCR in the plasma membrane. When a ligand (signal) binds the GPCR, GDP is exchanged for GTP on the Gα subunit and the Gβγ and Gα subunits dissociate. Free Gα-GTP and Gβγ subunits can then regulate downstream effectors, such as enzymes, ion channels, and other regulatory proteins. After GTP hydrolysis on the Gα subunit, the GDP-bound Gα protein reassociates with the Gβγ dimer and GPCR, allowing a new cycle of signaling (Li et al., 2007).

In filamentous fungi, there are three different families of Gα subunits, termed Group I, II, and III (Li et al., 2007). Group I and III Gα subunits are highly conserved among many filamentous fungi. Group I proteins are involved in multiple pathways, including pheromone response and mating, nutrient sensing, and pathogenesis. Group III proteins have been demonstrated to function in sexual spore development and in nutrient sensing related to cAMP-dependent pathways. Compared to Group I and III subunits, Group II has less obvious roles in filamentous fungi (Kays and Borkovich, 2004). However, a Group II protein is required for optimal growth on poor carbon sources and plays a compensatory role during cell growth, sexual development, and asexual sporulation in *N. crassa* (Li and Borkovich, 2006).

Most filamentous fungi possess a single Gβ and Gγ subunit, although more than one subunit is present in the genomes of several fungi, such as *Rhizopus oryzae*, *Cryptococcus neoformans*, *Coprinus cinereus*, and *Podospora anserina* (Li et al., 2007). In aggregate, evidence suggests that Gβ subunits are required for complex formation and/or stabilization of Gα and Gγ proteins and regulation of MAPK- and cAMP-dependent pathways during mating and pathogenesis (Krystofova and Borkovich, 2005; Yang et al., 2002).

The genomes of filamentous fungi contain genes encoding proteins other than canonical Gβ subunits that can form complexes with G proteins to regulate signal transduction. For example, a Gβ-like RACK1 homologue has been demonstrated to interact with Gα and Gγ proteins to regulate cAMP levels during formation of the virulence factors melanin and capsule in *C. neoformans* and to participate in protoperithecium formation and cross-pathway control of amino acid synthesis in *N. crassa* (Muller et al., 1995; Palmer et al., 2006).

As mentioned above, heterotrimeric G proteins associate with, and are regulated by, GPCRs that are present on the plasma membrane (Fig. 1). GPCRs contain seven-transmembrane (7-TM) helices connected by intracellular and extracellular loops. At least nine classes of GPCR genes have been enumerated to date in filamentous fungal genomes (Li et al., 2007; Xue et al., 2008). GPCRs homologous to pheromone receptors from yeasts have been shown to regulate mating in both ascomycete and basidiomycete fungi (Li et al., 2007). A predicted GPCR similar to cAMP receptor-like proteins from the protist slime mold *Dictyostelium discoideum* was characterized for its role during fruiting body development in *N. crassa* and during vegetative growth and conidiation in *Trichoderma atroviride* (Brunner et al., 2008; Krystofova and Borkovich, 2006). Proteins related to the carbon-sensory GPCR Gpr1p from *S. cerevisiae* function during sensing of carbon sources and amino acids in *N. crassa* and *C. neoformans*, respectively (Li and Borkovich, 2006; Xue et al., 2006). The 7-TM protein PTH11 is required for virulence in the rice pathogen *Magnaporthe grisea* (Kulkarni et al., 2005). Filamentous fungi contain a large number of predicted GPCRs homologous to PTH11, but functions for these are currently unknown (Li et al., 2007). In addition, cellular roles have not yet been revealed for several other classes of predicted GPCRs in filamentous fungi, including microbial opsins, Stm1-related proteins, human mPR-like receptors, rat growth hormone releasing factor-related proteins, and proteins similar to *Arabidopsis thaliana* AtRGS1 (for reviews, see Li et al., 2007, and Xue et al., 2008).

An increasing number of studies implicate other proteins, in addition to GPCRs, that regulate Gα, Gβ, and Gγ subunits in filamentous fungi. As GAPs, regulator of G protein signaling (RGS) proteins accelerate the GTPase activity of Gα subunits and negatively control Gα protein-mediated signaling (Fig. 1). Many filamentous fungi have more than one RGS protein, and each RGS acts on one or more groups of Gα subunits during the regulation of conidiation, pathogenicity, and secondary metabolism (Liu et al., 2007; Segers et al., 2004; Yu, 2006).

Phosducin-like proteins (PhLPs) have been shown to regulate Gβγ in filamentous fungi and other eukaryotes (Kasahara et al., 2000; Seo and Yu, 2006). As observed in *Cryphonectria parasitica* and *A. nidulans*, loss of PhLP genes leads to phenotypes identical to those of mutants lacking Gβ subunits (Kasahara et al., 2000; Seo and Yu, 2006). The results from *C. parasitica* preceded later studies in mammals that established a role for PhLPs as chaperones that stabilize Gβ proteins, thereby facilitating formation of the Gβγ dimer (Kasahara et al., 2000; Seo and Yu, 2006).

MAPK SIGNALING PATHWAYS

MAPKs are serine/threonine protein kinases that are regulated by a variety of upstream factors, including Cdc42p, GPCRs, p21-activated kinases, and response regulators

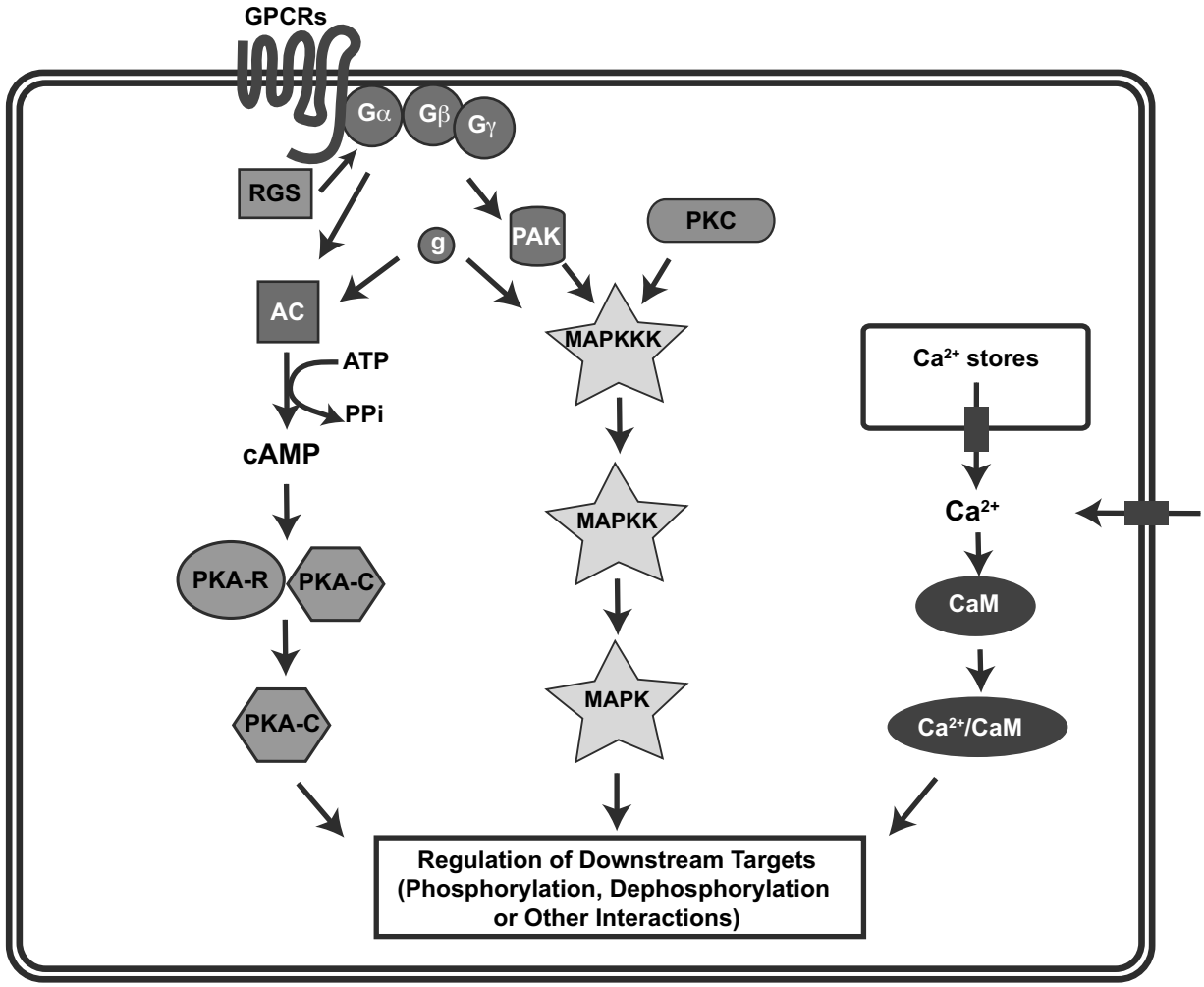

FIGURE 1 Overview of signal transduction pathways in filamentous fungi. Environmental signals are sensed by GPCRs or other unknown proteins, leading to regulation of downstream pathways. Activation of heterotrimeric and small G proteins can result in modulation of cAMP levels and MAPK pathways. In response to environmental signals, free Ca^{2+} transported into the cytoplasm from the external environment or from intracellular Ca^{2+} stores binds to the Ca^{2+} sensor calmodulin and other regulatory proteins. The Ca^{2+}-calmodulin complex regulates downstream targets, including the serine-threonine protein phosphatase calcineurin. Abbreviations: Gα, Gα subunit; Gβ, Gβ subunit; Gγ, Gγ subunit; g, small G proteins; PAK, p21-activated kinase; PKC, protein kinase C; AC, adenylyl cyclase; PKA-R, protein kinase A regulatory subunit; PKA-C, protein kinase A catalytic subunit; CaM, calmodulin.

from two-component regulatory systems (see below). MAPK modules operate in a three-tier, sequential phosphorylation cascade to regulate cellular processes. Within the MAPK module, the MAPK kinase kinase (MAPKKK) is first activated by phosphorylation. The MAPKKK then phosphorylates/activates the MAPK kinase (MAPKK), which in turn phosphorylates/activates the MAPK. The MAPK ultimately controls a variety of processes, including the cell cycle, mating, morphogenesis, and pathogenesis (Fig. 1). Three MAPK modules homologous to those involved in the pheromone response (Ste11p/Ste7p/Fus3p or Kss1p), osmoregulation (Ssk2/22p or Ste11p/Pbs2p/Hog1p), and cell wall integrity (Bck1p/Mkk1/2p/Slt2p) in *S. cerevisiae* are highly conserved in filamentous fungal genomes. Among the three cascades, the one that is most similar to Fus3p/Kss1p is the best characterized in filamentous fungi and has been shown to regulate the pheromone response, development, and pathogenicity (for a review, see Zhao et al., 2007). Hog1-like MAPK pathways have been implicated in the response to osmotic and oxidative stresses in many filamentous fungal species (for a review, see Zhao et al., 2007). However, roles for the Hog1p-related pathway in pathogenicity vary among filamentous fungi (Dixon et al., 1999; Segmuller et al., 2007). As observed in *S. cerevisiae*, the Slt2p-related MAPK cascade functions in the control of cell wall integrity in many filamentous fungi, although this is not the case in *Botrytis cinerea* and *Collectotrichum lagenarium* (Kojima et al., 2002; Rui and

Hahn, 2007). The Slt2p-related pathway also regulates vegetative growth, hyphal morphology, conidiation, and pathogenicity in several species (for a review, see Zhao et al., 2007) and has been recently shown to influence secondary metabolism in *N. crassa* (Park et al., 2008).

Many transcription factors that control gene expression in filamentous fungi are downstream targets of MAPKs. Ste12p-like transcription factors have frequently been implicated as downstream proteins controlled by the Fus3p/Kss1p-homologous MAPK (for a review, see Zhao et al., 2007). In *Ustilago maydis*, the high-mobility group domain transcription factor Prf1p is regulated by Fus3p/Kss1p-like MAPK (Kaffarnik et al., 2003). Finally, transcription factors homologous to Rlm1p in *S. cerevisiae* have been shown to function downstream of the Slt2p-like MAPK in *M. grisea* (Mehrabi et al., 2008).

Roles for Gβ subunits of heterotrimeric G proteins and the Ras GTPase as upstream regulators of Fus3p/Kss1p-related MAPKs have been demonstrated in several filamentous fungi during mating and pathogenesis (Lee and Kronstad, 2002; Wang et al., 2000). Overexpression of the Fus3p/Kss1p-like MAPK suppresses the mating defect of a Gβ mutant in *C. neoformans* (Wang et al., 2000). Both Ras2 and Ubc3 (MAPK) in *U. maydis* function in the pheromone response pathway, and constitutive activation of Ras2 induces pseudohyphal growth in the wild type but not in *fuz7* (MAPKK) and *ubc3* (MAPK) mutants (Lee and Kronstad, 2002). The Ste50p-related adapter protein physically interacts with the Ste11p-like MAPKKK and is required for activation of the Fus3p/Kss1p homologous MAPK in *M. grisea* and *U. maydis* (Klosterman et al., 2008; Park et al., 2006). Finally, response regulators from two-component regulatory systems have also been shown to regulate Hog1p-like cascades in filamentous fungi (Jones et al., 2007; Yoshimi et al., 2005; see below).

cAMP SIGNALING

cAMP acts as a second messenger that regulates signaling pathways in filamentous fungi and other eukaryotes. The enzyme adenylyl cyclase converts ATP to cAMP and pyrophosphate. Various proteins are implicated in the activation of adenylyl cyclase, including cyclase-associated protein, heterotrimeric G proteins, and small G proteins (Kozubowski et al., 2009; Lee et al., 2003). Intracellular cAMP binds to the regulatory subunits of PKA, and this association triggers dissociation of the regulatory and catalytic subunits, leading to activation of the freed catalytic subunits. Activated PKA then phosphorylates various protein targets that are critical for fungal growth, development, and pathogenesis (Kozubowski et al., 2009; Lee et al., 2003).

Perhaps the best-characterized function for the cAMP signaling pathway in both yeasts and filamentous fungi is nutrient sensing (Li et al., 2007; Xue et al., 2006). Sensing of carbon source or amino acids by a GPCR activates the coupled Gα subunit of a heterotrimeric G protein that in turn mediates transfer of the signal to adenylyl cyclase, leading to regulation of cAMP levels (Li and Borkovich, 2006; Xue et al., 2006). In addition to nutrient sensing, many cellular processes such as asexual sporulation, mating, pathogenesis, and secondary metabolism are also influenced by cAMP levels (Lee et al., 2003; Li et al., 2007; Shimizu et al., 2003). Finally, cross-talk between the cAMP-dependent pathway and MAPK cascades has often been observed, particularly during pathogenic development (for reviews, see Kozubowski et al., 2009, and Lee et al., 2003).

TWO-COMPONENT REGULATORY SYSTEMS

Two-component systems have been identified in bacteria, archaea, plants, protists, and fungi (Wolanin et al., 2002). Two-component regulatory systems are major signal transduction pathways in filamentous fungi (Fig. 2). "Two-component" refers to the two different protein domains present on proteins in this phosphorelay system: histidine kinase (HK) and response regulator (RR) domains. The HK is autophosphorylated (using ATP) in response to an environmental signal. The phosphate from the HK is then transferred to an RR domain present on either the same or a different protein. In filamentous fungi and yeasts, HKs are of the hybrid type, containing both HK and RR domains. In these systems, a histidine phosphotransferase (HPT) accepts phosphate from the RR domain on the hybrid HK (HHK). The phosphate on the HPT protein is ultimately transferred to an RR domain on a terminal RR protein. The phosphorylation status of the RR determines its regulatory effect on downstream effectors, such as MAPK cascades and/or gene transcription.

The sequenced genomes of several filamentous fungi reveal that most contain two or three RR protein-encoding genes, but only one for HPT proteins. The number of RR and HPT genes in filamentous fungi and yeasts is similar (Borkovich et al., 2004). However, the number of HHK protein genes in filamentous fungi is greater than that in sequenced yeasts (1 to 3) and ranges from 10 to 20. The HHKs of filamentous fungi have been categorized into 11 classes, according to the sensor domains present at the amino terminus (Catlett et al., 2003). Some of the HHKs contain one or more PAS (acronym for Per/Arndt [arcyl-hydrocarbon receptor nuclear translocator]/Sim [single-minded protein]) domains; PAS domains are thought to be involved in sensing redox potential, cellular oxygen, energy, and light and are found in proteins regulating hypoxia responses and circadian rhythms (Catlett et al., 2003; Taylor and Zhulin, 1999). GAF (acronym for cGMP phosphodiesterase/adenylyl cyclase/Fhl) domains bind to cyclic GMP and chromophores (Aravind and Ponting, 1997; Catlett et al., 2003). The phytochromes are known for their role in light sensing (Galagan et al., 2003). HAMP (acronym for HK, adenylyl cyclases, methyl-binding proteins, and phosphatases) domains are known to connect extracellular sensory and intracellular output domains in signaling proteins (Hulko et al., 2006).

In certain animal fungal pathogens, mutation of an HK or an RR component renders the strain avirulent (Du et al., 2005). This finding, along with the observation that two-component systems have not been found in any animal genome sequences, including those of humans, makes these pathways a potential target for the development of new antifungal agents (Wolanin et al., 2002).

The best-understood fungal two-component system is the Sln1p/Ypd1p/Ssk1p pathway in *S. cerevisiae* (Posas et al., 1996). When the HHK Sln1p becomes phosphorylated, it transfers its phosphate to the HPT protein Ypd1p and then onto the RR Ssk1p. When phosphorylated, Ssk1p is unable to activate the downstream MAPK pathway. When Sln1p is not phosphorylated, this leads to a buildup of Ssk1p in the unphosphorylated form and activation of the Ssk2p and Ssk22p MAPKKKs, ultimately leading to the phosphorylation of the Hog1p MAPK (Maeda et al., 1994). The Hog1p

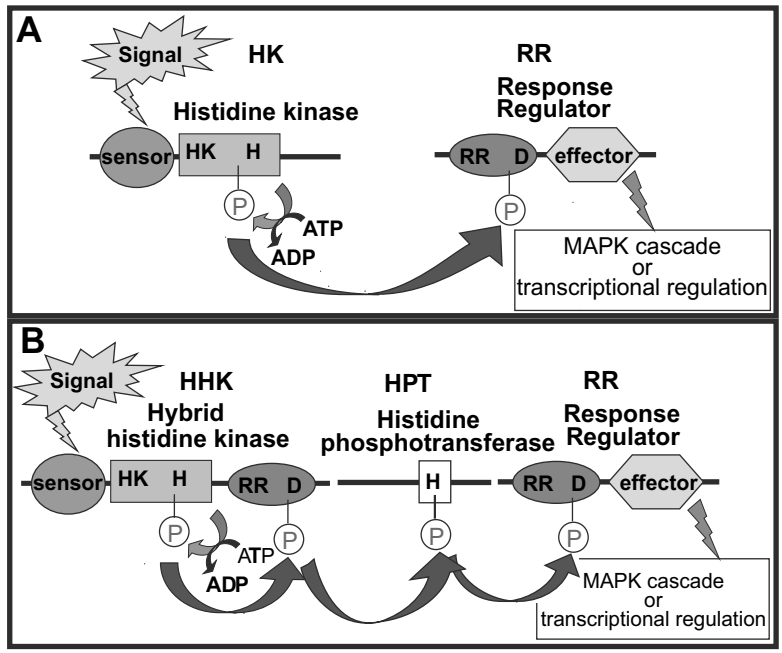

FIGURE 2 Two-component regulatory systems. (A) Two-component signaling pathway. In simple two-component signaling pathways the two components are an HK and an RR protein. The sensor domain(s) located at the N terminus of the HK protein senses environmental signals. In response to such a signal, a histidine residue (H) in the HK domain autophosphorylates, using ATP. The same phosphate (P) is then transferred to an aspartate residue (D) on the RR protein. The phosphorylation status of the RR determines how it regulates downstream pathways, such as MAPK cascades. Alternatively, the RR may act directly as a transcription factor. (B) Multicomponent phosphorelays. Multicomponent phosphorelays contain HHKs, possessing both an HK and an RR domain within the same protein. The sensor domain(s) of the HHK located at the N terminus senses an environmental signal(s) to regulate autophosphorylation of the HHK on a histidine residue in the HK domain. The phosphate is then transferred intramolecularly from the HK domain to an aspartate residue in the RR domain of the same HHK protein. The phosphorelay continues with transfer of the phosphate from the RR of the HHK to a histidine residue on an HPT. This same phosphate is then passed onto an aspartate residue on a terminal RR protein.

MAPK cascade regulates the expression of genes responsible for the synthesis of glycerol, a counter solute, which can help maintain internal osmolarity when the external environment is hyperosmotic (Maeda et al., 1994). The RR Skn7p, which is also regulated by the HHK Sln1p, has been found to be involved in cell wall assembly, regulation of the cell cycle, and the oxidative stress response (Brown et al., 1994; Morgan et al., 1997).

Mutation of certain two-component protein genes results in loss of osmotic and/or oxidative stress resistance in a variety of filamentous fungi. Filamentous fungi contain an HHK similar to Sln1p (the only HHK found in baker's yeast), but this protein has not been shown to regulate osmosensing. Instead, proteins similar to the HAMP domain-containing HHK OS-1 from *N. crassa* and RR Ssk1p homologues have been implicated in resistance to osmostress in several filamentous species. The HHK *os-1* or RR *rrg-1* (similar to *ssk-1*) from *N. crassa* and the *os-1*-like HHK, *fos-1*, from *Aspergillus fumigatus* all play a role in osmosensing (Clemons et al., 2002; Jones et al., 2007). In *N. crassa,* the RR *rrg-2* (similar to *S. cerevisiae skn-7*) is believed to regulate the oxidative stress response (Banno et al., 2007). In *C. neoformans* and *Candida albicans*, loss of the RR *ssk-1* yields both osmotic and oxidative stress phenotypes (Bahn et al., 2006; Chauhan et al., 2003). Of note, deletion of the RR *skn-7* results in sensitivity to hydrogen peroxide, but not to osmolarity in *C. albicans* (Singh et al., 2004).

A number of mutations in genes encoding proteins involved in two-component signaling, which are homologous to those involved in the *S. cerevisiae* osmotic stress response, have also been found to be important for virulence in filamentous fungi. Mutation of *fos-1* (the *os-1*-like HHK) from the lung pathogen *A. fumigatus* leads to a severe decrease in the level of virulence in mouse models (Clemons et al., 2002). The human pathogenic yeast *C. albicans* has three HHKs; deletion of any one HHK decreases virulence in mouse models (Selitrennikoff et al., 2001; Torosantucci et al., 2002; Yamada-Okabe et al., 1999). In addition, loss of the RR *ssk1* renders *C. albicans* avirulent (Du et al., 2005). Mutation of the HHK *tco-1* or the deletion of the RR *skn7* in *C. neoformans*, the causative agent of fungal meningitis, leads to reduced virulence (Bahn et al., 2005; Coenjaerts et al., 2006; Wormley et al., 2005). Mutation of two-component pathway genes has also been found to reduce virulence in plant pathogens. For example, mutation of the RR *rr-1* from *Fusarium graminearum* and deletion of Δ*bos-1* (*os-1*-like) from *Botrytis cinerea* significantly impaired the virulence of these organisms on their plant host (Goswami et al., 2006; Viaud et al., 2006).

Two-component pathways have also been implicated in resistance to fungicides. Iprodione, which is a dicarboximide fungicide, is used against plant pathogens such as *B. cinerea* (Ochiai et al., 2001; Pommer and Lorenz, 1995). The phenylpyrrole fungicide fludioxonil was recently approved for use on postharvest citrus (Kanetis et al., 2008). While *S. cerevisiae* is not sensitive to these fungicides, it is known that mutation of two-component (HK or RR) genes that control the osmotic stress response in fungicide-sensitive species can result in fungicide resistance (Motoyama et al., 2005; Zhang et al., 2002). Furthermore, the natural fungicide resistance of *S. cerevisiae* can be overcome by the introduction of the *os-1* HHK gene from *M. grisea* (Motoyama et al., 2005) or *Alternaria brassicicola* (Dongo et al., 2009). These results suggest that the OS-1 class of HHKs may play a direct role as a fungicide sensor in filamentous fungi.

CALCIUM SIGNALING

Environmental stresses or developmental cues promote Ca^{2+} transfer into the cell cytoplasm from the external environment or from intracellular stores (Fig. 1). Free calcium within the cell can act as a second messenger in eukaryotes. In filamentous fungi, calcium-mediated signaling is crucial for regulating stress responses, morphogenesis, and pathogenicity (Kraus and Heitman, 2003).

Calcium signaling pathway components are involved in regulation of intracellular free Ca^{2+} levels and Ca^{2+}-dependent signal transfer, leading to changes in gene expression. In eukaryotes, Ca^{2+} permeable channels, pumps, transporters, and phospholipase C (PLC) regulate the concentration of intracellular free Ca^{2+}. As shown in *N. crassa*, *M. grisea*, and *Aspergillus* spp., filamentous fungi possess multiple predicted Ca^{2+}-regulated channels, pumps, and transporter genes in their genomes (Bencina et al., 2009; Zelter et al., 2004). Since the number of these genes represents a significant proportion of the genome, calcium signaling holds promise as a major regulatory mechanism controlling cellular processes in filamentous fungi (Zelter et al., 2004).

In plants and animals, Ca^{2+} release into the cytoplasm is mediated by inositol triphosphate (IP_3) that binds to receptors on the surface of organelles containing intracellular calcium stores. IP_3 is synthesized by PLC. Fungal genomes lack apparent IP_3 receptors; however, filamentous fungi possess multiple PLC genes, and IP_3 is produced and involved in the activation of a Ca^{2+} channel in *N. crassa* (Silverman-Gavrila and Lew, 2002). The secondary messenger cAMP can also affect intracellular Ca^{2+} levels, through PKA-dependent activation of a Ca^{2+} channel, as shown in *A. niger* (Bencina et al., 2005).

Intracellular free Ca^{2+} exerts a major effect on downstream events through binding to the Ca^{2+} binding protein calmodulin (Chin and Means, 2000). In several plant and human pathogenic fungi, calmodulin is essential for full virulence, because it controls infection-related morphogenesis (Lee and Lee, 1998; Warwar et al., 2000). Ca^{2+}/calmodulin has been demonstrated to activate the serine-threonine protein phosphatase calcineurin (Fig. 1). Calcineurin is a heterodimer composed of catalytic and regulatory subunits, and the catalytic subunit is activated in response to stresses, as well as during fungal development and pathogenicity (Steinbach et al., 2006). Other known targets of Ca^{2+}/calmodulin in filamentous fungi include a kinase that regulates the circadian clock and a neutral trehalase that modulates conidiation (reviewed by Borkovich et al., 2004).

OTHER SENSORY PATHWAYS

Tor Pathway

As stated above, a GPCR/Gα/cAMP signaling pathway modulates nutrient sensing in filamentous fungi. Nutrient sensing is also regulated by the Tor kinase-mediated signaling pathway, which is highly conserved in all eukaryotes, from yeasts to humans (Rohde et al., 2008). Tor kinase is a member of the phosphatidylinositol 3-kinase superfamily and is inhibited by the antifungal agent rapamycin (Thomas and Hall, 1997). Treatment with rapamycin and nutrient deprivation lead to dramatic changes in cellular processes necessary for survival, and Tor signaling contributes to cell growth, translation, transcription, ribosome biogenesis, and autophagy in *S. cerevisiae* (Rohde et al., 2008). Under conditions of nitrogen starvation or rapamycin treatment, Tor kinase is inhibited, leading to dephosphorylation of a regulatory protein for PP2A-like protein phosphatase and activation of phosphatase activity (Beck and Hall, 1999). This leads to dephosphorylation and nuclear accumulation of the zinc-finger transcription factor, Gln3p, and expression of genes controlled by nitrogen catabolite repression (Beck and Hall, 1999). Accumulating evidence reveals coordination of Tor signaling with other pathways (Rohde et al., 2008). In particular, cAMP often regulates the same targets in parallel with Tor signaling in response to nutrient deprivation (Chen and Powers, 2006).

The observation that rapamycin inhibits the growth of several filamentous fungi suggests a role for Tor kinase in these organisms (Cruz et al., 1999; Dementhon et al., 2003). In contrast to *S. cerevisiae*, filamentous fungi appear to possess only one Tor kinase gene (e.g., *A. nidulans*, *P. anserina*, and *Fusarium fujikuroi* [Muthuvijayan and Marten, 2004; Pinan-Lucarre et al., 2006]). Accumulating evidence indicates that Tor signaling is required for filamentous development in dimorphic fungi and for expression of genes required for secondary metabolite biosynthesis in *F. fujikuroi* (Cutler et al., 2001; Teichert et al., 2006).

pH Regulation

Fungi can grow under a wide range of pH values due to the presence of the regulatory mechanisms that maintain intracellular pH homeostasis (Bencina et al., 2009). Intracellular pH level is regulated by a machinery that includes K^+ and Na^+/H^+ transporters, Ca^{2+}/H^+ exchangers, proton/nutrient transporters, and proton ATPases. Similarities in the V-type and F-type proton ATPase complexes observed in *Aspergillus* spp. and yeasts suggest a universal requirement for these ATPase complexes in fungal pH regulation (Bencina et al., 2009). In contrast, as demonstrated in *Aspergillus* spp., more cation/proton exchangers and P-type proton ATPases are present in filamentous fungi than in *S. cerevisiae*, suggesting more complex regulation of pH sensing in filamentous species (Bencina et al., 2009).

Regulation of gene expression by ambient pH has been extensively studied in the filamentous fungus *A. nidulans*. In *A. nidulans*, a zinc finger transcription factor, PacC, plays a major role in this regulation (for a review, see Penalva et al., 2008). PacC is in an inactive form under acidic conditions, and alkaline pH transforms PacC to an active form by triggering its proteolysis. The alkaline pH signal is thought to induce endocytosis of a plasma membrane complex that includes the 7-TM helix protein PalH (probable pH sensor)

and the arrestin protein PalF, resulting in an endosomal membrane complex on the surface of the endosome. In the endosome, the transferred signal activates the cysteine protease PalB to initiate proteolysis of inactive PacC that has been recruited to endosomal membrane by PalA, a component of the endosomal sorting complex required for transport. The first proteolysis generates a PacC form with an open conformation that is susceptible to further proteolysis. After a second round of proteolysis by the proteasome, the processed PacC transcription factor can activate genes expressed under alkaline conditions and repress genes expressed under acidic conditions (Penalva et al., 2008).

One of the important biological functions of PacC-mediated pH regulation in filamentous fungi is control of fungal pathogenesis, as demonstrated in phytopathogenic species. PacC-homologous transcription factors in these fungi have been demonstrated to regulate full fungal virulence in a positive or negative manner (Caracuel et al., 2003; Rollins, 2003; You et al., 2007).

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REPLICATION AND EXPRESSION OF THE GENETIC CODE

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6

Mitotic Cell Cycle Control

COLIN P. C. DE SOUZA AND STEPHEN A. OSMANI

During cell division, eukaryotic cells must faithfully pass on their genetic information to daughter nuclei. The eukaryotic cell cycle comprises two main events, DNA replication (S phase) and DNA segregation, which are generally separated by the so-called gap phases, G_1 and G_2. The period of time comprising G_1, S phase, and G_2 is called interphase, while the relatively short period of time in which the DNA is segregated is called mitosis. The cell cycle has been extensively studied at the ultrastructural, biochemical, and genetic levels in many model systems, from yeasts to human tissue culture cell lines. Model yeast systems have had an enormous impact on our current understanding of how the cell cycle works, and it is now clear that many cell cycle regulatory networks are conserved in humans. In addition, findings for filamentous fungi have also made significant contributions to the understanding of how the cell cycle is regulated. This chapter focuses on our current understanding of how control of the mitotic phase of the cell cycle is achieved in filamentous fungi.

FUNGAL MITOSIS

Survival of filamentous fungi requires exploration by rapid polarized growth to find nutritional requirements, as well as the production of large numbers of asexual and/or sexual spores that can lie dormant until suitable conditions trigger germination. These scenarios require rapid nuclear proliferation, and therefore, the cell cycle of filamentous fungi is completed within a relatively short time in comparison to a typical mammalian cell cycle, which takes close to 24 h. For example, in *Aspergillus nidulans* one cell cycle takes about 2 h, although nuclei are in mitosis for only 5 min (Bergen and Morris, 1983). These rapid nuclear divisions are orchestrated by the cell cycle machinery and must be coordinated with growth and development. In different fungal species, this can be achieved in different ways. For example, while the syncytia of both *Ashbya gossypii* and *A. nidulans* contain multiple nuclei, in *A. gossypii* nuclear divisions occur asynchronously, while in *A. nidulans* nuclear divisions occur in a parasynchronous manner (Gladfelter, 2006).

The mitotic spindle is the structure upon which eukaryotic chromosomes are segregated and is comprised of microtubules emanating from the spindle poles and connecting to the kinetochores of the chromosomes (Fig. 1). While nuclear envelope (NE) breakdown is a hallmark of higher-eukaryote mitosis, many fungi undergo a closed mitosis within an intact NE. Therefore, in many fungi, the NE can also be considered to be an important part of the mitotic apparatus. In fungi, the mitotic microtubule organizing center is the spindle pole body, while in mammalian cells the centrosomes perform this function. Microtubules are comprised of heterodimers of α- and β-tubulin and have a plus end and a minus end (Oakley, 2004). In a spindle, the minus ends are nucleated from the spindle pole bodies and attach to kinetochores, to form kinetochore microtubules. In addition, microtubules emanating from opposite poles interdigitate their plus ends to form interpolar microtubules. Mitosis is in large part achieved by the dynamic nature of microtubules, and indeed the different stages of mitosis, termed prophase, metaphase, anaphase, and telophase, are defined by the characteristic changes in microtubule organization which take place during mitotic progression. Many of these changes are orchestrated by microtubule motor proteins of the dynein and kinesin families.

Studies with filamentous fungi have had a large impact on our understanding of microtubule and motor protein function (for reviews, see Oakley, 2004; Fischer et al., 2008; Xiang and Fischer, 2004; and Xiang and Plamann, 2003; see also chapter 16). Many of these studies were carried out with *A. nidulans*, in which α- and β-tubulin mutants that affected microtubule function were first isolated (Oakley, 2004). This subsequently led to the cloning of the prototypical γ-tubulin (Oakley and Oakley, 1989), which is now known to have universal roles in nucleating microtubules not only from fungal spindle pole bodies but also from mammalian centrosomes. In addition, some of the first mutants encoding microtubule motor proteins were isolated in *A. nidulans*, and roles for such motor proteins have been well studied in other model fungal systems

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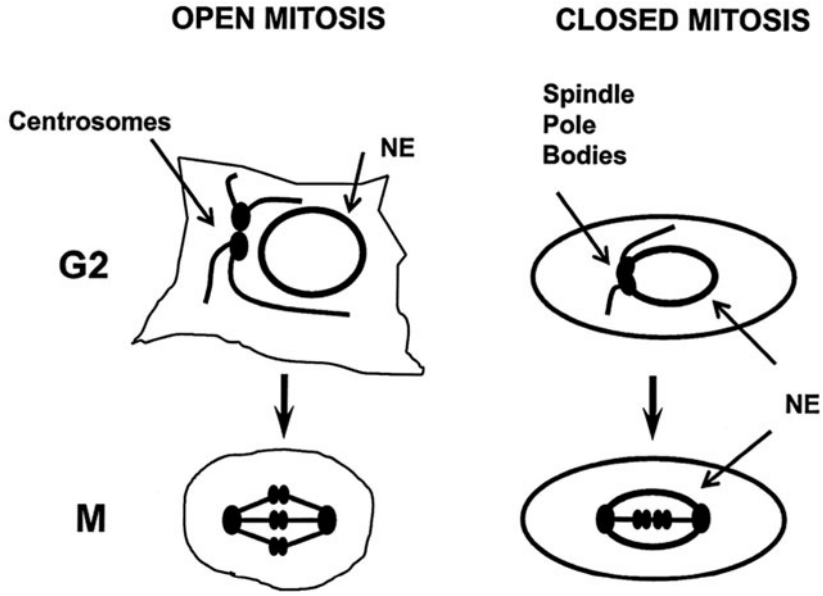

FIGURE 1 Schematic showing mammalian open mitosis and fungal closed mitosis. The NE breaks down during open mitosis so that microtubules nucleated from the cytoplasmic centrosomes can attach to kinetochores. During closed mitosis the spindle pole bodies are in the NE and nucleate spindle microtubules that attach to kinetochores within nuclei.

such as *Neurospora crassa* (Xiang and Fischer, 2004; Xiang and Plamann, 2003).

It has been known for quite some time that there are many variations in the structural features of fungal mitosis (Heath, 1980). Here we describe the more general features and discuss some variations in later sections. During interphase, microtubules are cytoplasmic and nuclear DNA is uncondensed. This is similar in mammalian cells and fungal cells, although while mammalian centrosomes are cytoplasmic, fungal spindle pole bodies are generally located within the NE (Fig. 1). By G_2, both the DNA and spindle pole bodies have been duplicated, although segregation of either has not occurred. Fungal spindle formation begins at the G_2/M transition, when cytoplasmic microtubules begin to depolymerize and nuclear microtubules form from the nuclear face of the spindle pole bodies and attach to the kinetochores. At this stage, prophase, DNA condensation also occurs and spindle pole body separation begins. Metaphase then follows, although in filamentous fungi a distinct metaphase plate is not formed. Chromosome segregation begins in anaphase A, with the dissolution of sister chromatid cohesion and their initial movement towards the spindle poles. Spindle elongation and segregation of the NE then occurs in anaphase B and telophase. However, it is important to note that because of the multinucleate nature of fungal hyphae, cytokinesis does not occur in every cell cycle, and when it does, cells do not typically physically separate.

It is the daunting job of cell cycle regulators to precisely orchestrate the dramatic morphological changes which take place during mitosis in both a temporal and a spatial manner. These regulators must also coordinate mitosis with the rest of the cell cycle such that mitosis and DNA replication alternate and occur once, and only once, each cell cycle. Also in the job description for the cell cycle regulator is the ability to engage checkpoints to prevent cell cycle progression in response to defects in DNA replication, genome integrity, or spindle function. The use of genetic screens in lower eukaryotes has defined many of these cell cycle regulatory mechanisms.

GENETIC APPROACHES TO IDENTIFY CELL CYCLE-SPECIFIC GENES

While by the early 1970s the morphological aspects of mitosis had been described for many organisms, including a vast array of filamentous fungi (for reviews, see Aist and Morris, 1999, and Heath, 1980), the genes regulating the cell cycle were unknown. Researchers with great foresight turned to the genetics of simple eukaryotes to identify mutants with cell cycle defects. Genetic screens performed with budding yeast by Leland Hartwell, fission yeast by Paul Nurse, and *A. nidulans* by Ron Morris aimed to identify temperature-sensitive cell cycle mutants. While the cell cycle status of such mutants was monitored by bud size in the case of *Saccharomyces cerevisiae* and cell size in the case of *Schizosaccharomyces pombe*, Morris scored the mitotic events of chromosome condensation and spindle formation in *A. nidulans* (Morris, 1976b). Mutants that arrested in interphase were termed "never in mitosis" (*nim*), while those which accumulated in mitosis were termed "blocked in mitosis" (*bim*). In addition, because these phenotypes were scored in multinucleate hyphae, mutants with defects in septation (*sep* mutants) and nuclear migration (*nud* mutants) were also identified. This screen identified 26 *nim* mutants, 9 *bim* mutants, 5 *sep* mutants, and 5 *nud* mutants (Morris, 1976b). Subsequent genetic analysis determined that multiple alleles of only some genes were identified, suggesting that the screen was not saturating. The cell cycle defects that resulted in a *nim* phenotype occurred in 23 genes, and those resulting in a *bim* phenotype occurred in 6 genes. While this screen laid the foundation for cell cycle analysis in the model filamentous fungus *A. nidulans*, it is important to recognize the pioneering work of Guido Pontecorvo and Etta Kafer and their colleagues, who helped

establish the genetic utility of this organism (Pontecorvo, 1953; Pontecorvo and Kafer, 1958).

The identification of *nim* and *bim* mutants proved to be of great value for the study of the cell cycle even before the establishment of molecular cloning allowed their identification. Several of these temperature-sensitive alleles caused a cell cycle arrest that was readily reversible upon return to permissive temperature (Bergen and Morris, 1983; Morris, 1976a; Oakley and Morris, 1983), allowing the generation of cell cycle synchronized populations. Further dissection of the cell cycle was possible by determining where in interphase *nim* mutants arrested. These studies utilized hydroxyurea (HU) to cause a reversible S-phase arrest and also defined the length of the different cell cycle phases in *A. nidulans* (Bergen et al., 1984).

In addition to the above and other genetic screens for temperature-sensitive mutants (Orr and Rosenberger, 1976a, 1976b), screens for aneuploidy-generating mutants (Upshall and Mortimore, 1984) and DNA damage-sensitive mutants (Shanfield and Kafer, 1969; Goldman and Kafer, 2004) have also identified genes important for cell cycle regulation in *A. nidulans*. The use of secondary genetic screens to identify cell cycle regulators has also been productive. These include screens for extragenic suppressors (De Souza et al., 2006; Holt and May, 1996; Kraus and Harris, 2001; McGuire et al., 2000; Wu et al., 1998), synthetic lethal interactions (Efimov and Morris, 1998), copy number suppressors (De Souza et al., 2003; Xiang et al., 1995, Ukil et al., 2008), and two-hybrid interactors (Davies et al., 2004; Osmani et al., 2003). Of these, screens to identify extragenic suppressors of *nim*, *bim*, or other temperature-sensitive mutants have been most utilized. We discuss some of the genes identified in these extragenic suppressor screens in subsequent sections of this chapter but would like to highlight one particular extragenic suppressor screen. This screen, performed by Berl Oakley's lab, was for extragenic suppressors of the *benA*33 β-tubulin mutant and led to the discovery of *mipB* (microtubule interacting protein), which encoded a new type of tubulin now known by its universal name, γ-tubulin (Oakley and Oakley, 1989). This landmark discovery paved the way for the discovery of γ-tubulin ring complexes, which are now known to be the microtubule nucleation sites within spindle pole bodies and centrosomes (reviewed by Job et al., 2003). Further analysis of *A. nidulans* γ-tubulin mutants, generated by alanine-scanning mutagenesis, has uncovered mitotic roles for this protein independent of its roles in microtubule nucleation (Jung et al., 2001; Prigozhina et al., 2004). This work highlights the importance of forward genetics for gene discovery.

GENOMIC APPROACHES FOR CELL CYCLE GENE IDENTIFICATION

Primary and secondary genetic screens have given great insight into cell cycle regulation in *A. nidulans* as well as other organisms. However, these screens have not identified all cell cycle regulators. For example, one notable omission from the Morris screen was the Cdk1 kinase, which was known to be essential for mitotic entry in other organisms. A reverse-genetic approach was used to isolate the gene encoding Cdk1, *nimX*^{Cdk1}. As this was done in the pregenomics era, *nimX*^{Cdk1} cloning was achieved by PCR using degenerate oligonucleotides (Osmani et al., 1994). Reverse genetics were further applied to generate temperature-sensitive alleles of *nimX*^{Cdk1} based on known temperature-sensitive point mutations identified in *S. pombe cdc2* (the prototypic Cdk1) (Osmani et al., 1994). This strategy worked because of the high level of sequence and functional conservation between Cdk1 orthologues and allowed the roles of *nimX*^{Cdk1} in both the G_1/S and G_2/M transitions to be defined.

The recent availability of the complete genomic sequence of *A. nidulans*, as well as many other filamentous fungi (Dean et al., 2005; Dietrich et al., 2004; Galagan et al., 2003, 2005; Kamper et al., 2006), has greatly streamlined gene manipulations such as deletion or tagging. Combined with improved gene-targeting techniques, these strategies will no doubt garner major insight into the cell cycle as well as other processes in filamentous fungi. However, we should avoid the temptation of using reverse genetics alone, as the most significant findings have, and will likely continue to, come from novel gene discovery.

The genomics era enables the use of global approaches to study gene function, as has occurred with great success in *S. cerevisiae* (http://www.yeastgenome.org/). The simplest application of genomic data is to identify fungal orthologues of proteins with known mitotic functions to study their function in filamentous fungi. This approach, however, has its limitations, not the least of which is the reoccurring finding that even highly conserved proteins can have quite different functions in yeasts and in filamentous fungi. However, there is currently great potential for targeted genomic approaches, such as targeting all protein phosphatases (Son and Osmani, 2009) or all protein kinases to define their roles in mitotic regulation. There is also an ongoing *N. crassa* large-scale deletion program (Colot et al., 2006), and it will be interesting to define what gene deletions cause defects in mitosis by using this unbiased approach. Proteomic approaches also have great power to identify new proteins that interact with mitotic regulators. Most secondary genetic screens, such as copy number suppressor and extragenic suppressor screens, tend to identify proteins that physically interact with the initial target protein. The biochemical equivalent of such screens is protein complex purification. The power of such approaches has recently been nicely demonstrated by purification of the VeA protein using a TAP-tag approach, which linked this global developmental regulator to the global secondary metabolism regulator LaeA (Bayram et al., 2008). An S-tag affinity approach has also been successfully employed to purify *A. nidulans* nuclear pore complex (NPC) proteins together with their associated proteins (Liu et al., 2009). This led to the discovery of two NPC proteins that were previously thought to be vertebrate specific and were not readily identifiable based on sequence homology alone. Compared to genetic screens, the affinity purification approach is very fast and has an advantage over genetic screens and 2-hybrid approaches as de facto proteins are known to physically interact, although not necessarily in a direct manner. Such approaches, in combination with targeted or genome-wide green fluorescent protein tagging and live cell imaging, has tremendous potential to revolutionize our understanding of fungal mitotic regulation and mitotic regulation in general.

REGULATION OF MITOTIC PROGRESSION THROUGH PROTEIN PHOSPHORYLATION

The work that defined the mechanism of mitotic regulation by the Cdk1 cyclin-dependent kinase in partner with mitotic B-type cyclins resulted in Paul Nurse, Leland Hartwell, and Tim Hunt being awarded a Nobel Prize in 2001. In addition to Cdk1/cyclin B, other classes of Cdks and cyclins have been demonstrated to drive different aspects of the cell cycle in humans. As a result, it has become clear that the regulation of protein phosphorylation through the cell cycle is at the heart of its regulation. In addition to Cdk1, other

classes of kinases are essential for mitosis, and work involving filamentous fungi has contributed to our understanding of protein phosphorylation during mitosis. As is the case for the Cdk family, simple eukaryotes generally only have one member of a mitotic protein kinase family, while multiple members are found in human cells. Regulators of mitotic phosphorylation include the mitotic kinases Cdk1/cyclin B, NIMA, Aurora, and Polo, as well as mitotic phosphatases.

Cdk1/Cyclin B

The role for Cdk1/cyclin B in promoting mitosis is universally conserved in eukaryotes (Fig. 2). To function, Cdk1 requires a cyclin binding partner. This complex is kept inactive in G_2 by Wee1 kinase-mediated inhibitory phosphorylations on adjacent threonine and tyrosine residues within the ATP-binding domain. Activation at the G_2/M transition is achieved by dephosphorylation of these residues by the dual-specificity Cdc25 phosphatase. The components of this regulatory network are present in the genomes of filamentous fungi, and in *A. nidulans*, *A. gossypii*, and *Ustilago maydis* experimental evidence suggests that these components function in an analogous manner (Harris, 2006; Helfer and Gladfelter, 2006; Perez-Martin et al., 2006). The *A. nidulans* orthologues of cyclin B (NIME) and Cdc25 (NIMT) were identified in Ron Morris's screen and subsequently cloned (O'Connell et al., 1992), while Cdk1 and Wee1 were found by reverse genetics (Osmani and Mirabito, 2004). However, in *A. nidulans*, activation of Cdk1 kinase activity alone is not sufficient to trigger mitotic entry.

NIMA

The phenotype of *nimA5* is one of the most strikingly reversible of all the *nim* mutants identified in *A. nidulans*. This mutation causes a late G_2 cell cycle arrest at restrictive temperature, which upon return to permissive temperature results in synchronous mitotic entry (Oakley and Morris, 1983). This defined *nimA* function as being essential for mitotic entry. Cloning of *nimA* by complementation of *nimA5* temperature sensitivity revealed that it encoded a protein kinase that was not a member of the Cdk family (Osmani et al., 1987, 1988b) but defined a new family of protein kinases. NIMA is tightly regulated during the cell cycle, and levels of both its mRNA and protein peak late in G_2, when its activity is maximal (Osmani et al., 1987, 1991a). Most surprising was the finding that at the *nimA5* G_2 arrest point, Cdk1/cyclinB was fully active (Osmani et al., 1991a). Therefore, NIMA has a role in promoting mitosis which is independent of Cdk1/cyclin B protein kinase activation. This role likely extends to other filamentous fungi, as *nimA* orthologues from *N. crassa* and *Magnaporthe grisea* can complement *A. nidulans nimA* mutants (Osmani et al., 1991a; Pu et al., 1995; Veneault-Fourrey et al., 2006). However, NIMA is itself regulated by protein phosphorylation and its full mitotic activity requires multiple phosphorylations which are dependent upon Cdk1/cyclin B (Ye et al., 1995). Therefore, mitotic entry in *A. nidulans* requires the coordinated activities of both NIMA and Cdk1/cyclin B (Fig. 3).

Insight into why *nimA* mutants arrest in G_2 with activated Cdk1/cyclin B came from study of the location of cyclin B. Although cyclin B normally accumulates in G_2 nuclei, it is excluded from nuclei in a *nimA* mutant G_2 arrest (Wu et al., 1998). This suggests a role for NIMA in allowing Cdk1/cyclin B access to its nuclear substrates. The identification of mutations in two different NPC proteins which suppress a *nimA1* G_2 arrest suggests that NIMA targets the NPC to facilitate nuclear entry of Cdk1/cyclin B (De Souza et al., 2003; Wu et al., 1998). We discuss in more detail how NIMA regulates changes in mitotic nuclear transport later in this chapter.

Further studies indicated that ectopic expression of NIMA was sufficient to promote the mitotic events of chromatin condensation and spindle formation, even in cells arrested either in S phase or without Cdk1 activity (Osmani et al., 1988a; Ye et al., 1995). The location of NIMA during the cell cycle is dynamic and fits nicely with its roles in regulating the NPC, chromatin condensation, and spindle function (De Souza et al., 2000, 2004). NIMA is cytoplasmic in G_2, and as cells enter mitosis, it becomes prominent at the nuclear periphery, where it displays a location similar to that of NPCs (De Souza et al., 2004). Following this, it enters the nucleus, where it has a role in phosphorylating the histone H3 serine 10 residue, an event implicated in chromatin condensation (De Souza et al., 2000). Subsequent to this, NIMA locates along the spindle in metaphase

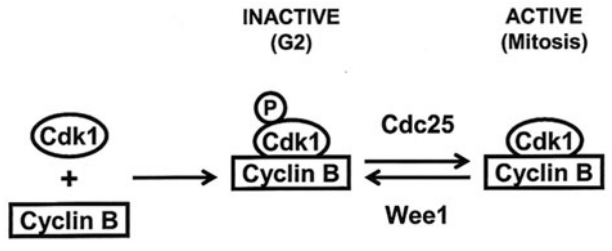

FIGURE 2 Regulation of Cdk1/cyclin B by tyrosine dephosphorylation. Cdk1/cyclin B activation requires dephosphorylation carried out by the Cdc25 phosphatase.

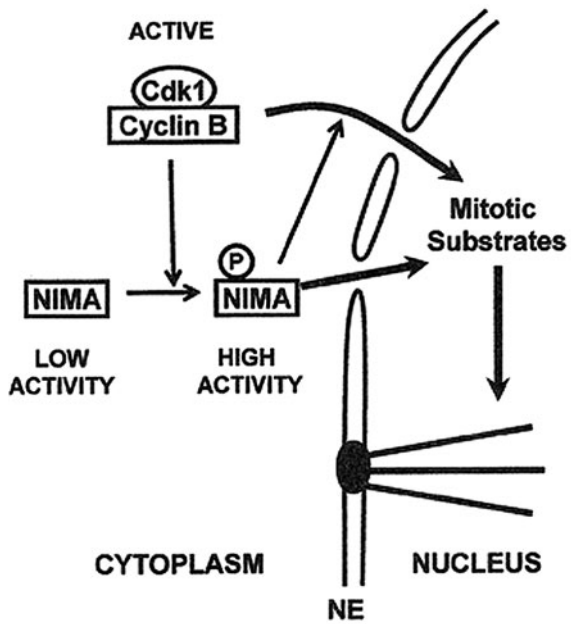

FIGURE 3 Mitotic entry requires both Cdk1/cyclin B and NIMA kinase activities in *A. nidulans*. Active Cdk1/cyclin B cannot enter the nucleus without NIMA activity. NIMA needs Cdk1/cyclin B-dependent phosphorylations to become fully active. Activation of both Cdk1/cyclin B and NIMA allows them to enter the nucleus and phosphorylate their nuclear substrates, thereby triggering mitosis.

and accumulates at the spindle poles late in mitosis before it is degraded during mitotic exit (De Souza et al., 2000). This dynamic location pattern suggests that NIMA has functions at different stages of mitosis and interacts with many proteins that may also be substrates. One of these interacting proteins is TINA, which was identified as a NIMA interacting protein in a yeast two-hybrid screen (Osmani et al., 2003). TINA locates to the spindle pole body during mitosis, and its deletion interferes with proper microtubule function at the spindle pole body, a function conserved in *S. pombe* (Toya et al., 2007).

Given the essential mitotic roles of the prototypic NIMA kinase in *A. nidulans*, there has been much interest in defining the mitotic roles of NIMA-related kinases in other organisms. Although the *S. cerevisiae* NIMA orthologue Kin3 is not essential, a temperature-sensitive allele has been generated which prevents phosphorylation of its mitotic substrates (Chen et al., 2002), causing lethality. In *S. pombe*, the Fin1 NIMA orthologue was identified in a genetic screen for mitotic mutants, and although not essential for mitosis, Fin1 does play roles during mitotic progression (Grallert et al., 2004; Grallert and Hagan, 2002; Krien et al., 1998, 2002). In human cells and other higher eukaryotes, NIMA-related kinases have been termed NEKs (acronym for NIMA-related kinase). Determining NEK function is complicated by the presence of 11 NEK genes, which in some cases encode splice variants (O'Regan et al., 2007). However, initial studies indicated that ectopic expression of *A. nidulans* NIMA or *S. pombe* Fin1 could induce mitotic events in human cells (Krien et al., 1998; Lu and Hunter, 1995; O'Connell et al., 1994), while expression of dominant negative NIMA constructs caused a G_2 arrest (Lu and Hunter, 1995; Lu and Means, 1994). This suggested that human NEKs have roles in mitotic regulation. Indeed, of the 11 human NEKs, four have been implicated in regulating mitotic events, while others have more specialized roles in regulating cilia function (O'Regan et al., 2007; Quarmby and Mahjoub, 2005).

The Aurora and Polo Mitotic Kinases

In addition to Cdk1 and NIMA, other kinases are involved in mitotic regulation. Of these, members of the Aurora and Polo kinase families have been most studied (Nigg, 2001). Although orthologues of these kinases are present in fungal genomes, to date little is known about their function. However, it would be expected that fungal Aurora and Polo kinases have functions in regulating mitosis, and this has been demonstrated in *A. nidulans* (Bachewich et al., 2005; C. P. De Souza and S. A. Osmani, unpublished observations).

Therefore, progression through mitosis requires a complex network of mitotic phosphorylations, which are primarily driven by the Cdk1, NIMA, Polo, and Aurora kinases. While fungal genomes generally encode only one of each of these mitotic kinases, higher eukaryote genomes encode multiple Cdks, NIMA-related kinases, Polo kinases, and Aurora kinases, which have functions in mitosis (Nigg, 2001; O'Connell et al., 2003; O'Regan et al., 2007; Petronczki et al., 2008). This likely reflects the increased complexity of cell cycle regulation in higher eukaryotes, along with specialized functions in different cell or organ types. Consistent with this, in mice, Cdk1 is sufficient to drive the cell cycles during embryonic development, but more specialized cell types require other Cdks (Santamaria et al., 2007). Not mutually exclusive to this is the likelihood that multiple roles can be performed by a single mitotic kinase in lower eukaryotes, but that in higher eukaryotes, these different roles are accomplished by distinct members of the kinase family.

Mitotic Phosphatases

Given that mitotic entry and progression are driven by phosphorylation, it stands to reason that there should exist phosphatases that dephosphorylate kinase substrates. Supporting this, *A. nidulans bimG* is a type I protein phosphatase whose mutation causes a mitotic arrest with elevated levels of phosphorylated proteins recognized by the MPM-2 phospho-specific antibody (Doonan and Morris, 1989). BIMG locates to the spindle pole bodies, nucleolus, and septa, suggesting it may dephosphorylate substrates at several different mitotically relevant locales (Fox et al., 2002).

Another phosphatase that regulates mitotic exit and cell division is the Cdc14 phosphatase, which is a component of the mitotic exit network/septation initiation network in budding and fission yeasts, respectively (Wolfe and Gould, 2005). Interestingly, in *Candida albicans*, Cdc14 locates to the bud neck during yeast-like budding but does not locate to septa during hyphal growth (Clemente-Blanco et al., 2006). Further, deletion of *C. albicans* Cdc14 causes defects in mitotic exit and morphogenesis. This suggests that Cdc14 has roles in mitotic exit in this organism but that these roles differ depending upon the developmental stage.

CELL CYCLE REGULATION THROUGH REGULATED PROTEIN DEGRADATION

As mitotic entry is primarily driven by phosphorylation regulated at the level of mitotic kinase activation, mitotic exit requires the inactivation of these kinases. Cells achieve this through the regulated degradation of either the mitotic kinases themselves or their regulatory subunits. For example, the defining characteristic of the prototypic Cdk1 cyclin binding partner, cyclin B, is its interphase accumulation followed by rapid degradation during mitotic exit (Murray et al., 1989; Potapova et al., 2006). Degradation of cyclin B completely inactivates Cdk1, as it no longer has a cyclin partner. Cyclin B is targeted for its proteasome-mediated degradation by ubiquitination carried out by the anaphase-promoting complex (APC) (Fig. 4). The degradation of NIMA is also required for mitotic exit (Pu and Osmani, 1995), and this is also regulated by the APC (Fig. 4) (Ye et al., 1998). While turning off kinases by degradation may seem like an all-or-nothing approach, it has become clear that such degradation is both temporally and spatially regulated. Filamentous fungal studies have contributed to this field of research, which is still a very topical area of biology.

As the *bim* mutants isolated in *A. nidulans* cause a mitotic arrest, it would be predicted that they code for functions required for mitotic exit. Of these mutants, the *bimE7* mutation gave the tightest mitotic arrest and was of particular interest, as it was shown to be able to override either an S-phase HU arrest, or a *nim*A5 G_2 arrest, and allow cells to enter mitosis (Osmani et al., 1988a). This indicated that BIME had a function in interphase as well as mitotic exit and suggested that its interphase function may be to keep NIMA activity below the threshold required for mitotic entry. The cloning of *bimE* revealed that it encoded a large protein, but its sequence did not shed much light on how it functioned (Engle et al., 1990). Subsequently, Marc Kirschner's lab biochemically purified *Xenopus* APC and found that it contained orthologues of not only BIME but also BIMA (Peters et al., 1996). This defined BIME and

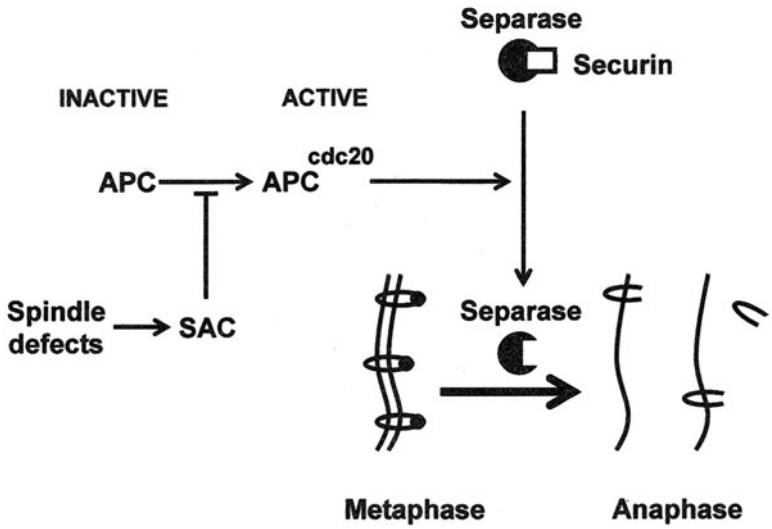

FIGURE 4 The APC regulates mitotic exit by triggering sister chromatid segregation. Cdc20 binds to and activates the APC. This allows the APC to ubiquitinate securin, targeting it for degradation by the proteasome. Securin degradation relieves inhibition of separase, which then degrades the Scc1 component of the securin ring complex. Spindle defects activate the SAC, which prevents Cdc20 binding to APC.

BIMA as components of the APC, consistent with the mitotic arrest caused by their loss of function. APC mutants have also recently been shown to arrest in late mitosis in *A. gossypii* (Gladfelter et al., 2007).

Additional studies have further explored the function of the *A. nidulans* APC components BIME^{APC1} and BIMA^{APC3} in interphase. Interestingly, while APC mutants can override a *nimA5* G_2 arrest, they cannot surpass the G_2 arrest caused by deletion of *nimA* (Ye et al., 1998). In addition, APC mutants cannot override a G_2 arrest caused by lack of Cdk1/cyclin B function (James et al., 1995; Lies et al., 1998). Therefore, both NIMA and Cdk1/cyclinB are required for mitotic entry, even in the absence of APC activity. Biochemically, loss of APC greatly increases Cdk1/cyclin B kinase activity (Ye et al., 1998). This increased Cdk1/cyclin B kinase activity presumably makes a *nimA5* G_2 arrest leaky, possibly by phosphorylation of NIMA5 protein. Morphological studies suggest that this leakiness may be manifested by the discontinuity of the NE observed in *nimA5 bimE7* double mutants (Osmani et al., 1991b). This would result in permeability of the NE, and the normal compartmentalization of Cdk1/cyclin B to the cytoplasm in a *nimA5* mutant (Wu et al., 1998) would be lost, allowing mitotic entry.

In addition to regulating levels of mitotic kinase activity, the APC also regulates sister chromatid dissolution during anaphase. It does this by targeting a protein called securin for degradation. This relieves the securin-mediated inhibition of a protease called separase, which in turn degrades Scc1, a component of the ring-like protein complex called cohesin, which holds sister chromatids together (Fig. 4) (Gruber et al., 2003). This elegant regulatory network, first defined in budding yeast (Uhlmann et al., 1999; Gruber et al., 2003), is also present in filamentous fungi, as indicated by the finding that *bimB* encodes the *A. nidulans* separase (May et al., 1992). In addition, other regulators of sister chromatid cohesion have been identified genetically in *A. nidulans*. One of the four subunits of the cohesin ring complex is encoded by *sudA*, which was identified as an extragenic suppressor of the *bimD6* temperature-sensitive mutation (Holt and May, 1996). The *bimD* gene encodes an orthologue of *Sordaria macrospora* Spo76 and human Pds5A/B, regulators of sister chromatid cohesion. Similar to the process that occurs in vertebrates, BIMD dissociates from chromatin during prophase (van Heemst et al., 2001). Interestingly, BIMD is also important for cell cycle regulation, as *bimD6* mutants transit the cell cycle faster than the wild type and BIMD overexpression leads to a G_1 arrest (van Heemst et al., 1999, 2001).

The APC is also targeted by cells to prevent mitotic exit in response to defective spindle formation by using a regulatory system called the spindle assembly checkpoint (SAC) (Fig. 4). This checkpoint was identified in budding yeast and monitors the attachment of microtubules to kinetochores (reviewed by Musacchio and Salmon, 2007). If microtubules are not correctly attached to all kinetochores in a bipolar manner, the SAC inhibits the activation of the APC (Fig. 4). This is achieved by the inhibitory binding of certain SAC proteins to APC activators such as Cdc20, thus inhibiting APC function. This is a universal mechanism in eukaryotes; components of the SAC are conserved in filamentous fungi, and mutants have been identified by both forward and reverse genetics in *A. nidulans* (Efimov and Morris, 1998; Prigozhina et al., 2004). Such SAC mutants display greatly enhanced sensitivity to drugs, such as benomyl, which interfere with spindle function and normally lead to SAC activation. These SAC mutants do not delay anaphase if correct microtubule attachments are not made and hence undergo lethal mitosis.

In general, the nuts and bolts view of mitotic regulation is that kinase activation triggers mitotic entry while the activation of protein degradation triggers mitotic exit. One study has revealed an unexpected relationship between these counteracting biochemical processes. In this work, rapid inactivation of the APC component BIMA^{APC3} in an asynchronous cell cycle population did not

cause a mitotic arrest as would be expected. Rather, rapid inactivation of $BIMA^{APC3}$ resulted in synchronous cell cycle oscillations of Cdk1/cyclin B and NIMA kinase activity (Ye et al., 1998). This indicates that $BIMA^{APC3}$ may be part of a cell cycle clock mechanism that can be reset upon its inactivation. Findings such as these are important, as we still do not understand how the cell cycle clock is regulated. For example, while the time taken to replicate or segregate DNA defines the length of S phase and mitosis, respectively, less is known about how the length of G_1 is regulated and almost nothing is known about how the duration of G_2 is controlled.

MODULATION OF NUCLEAR TRANSPORT DURING THE CELL CYCLE

Studies of cell cycle regulation utilizing filamentous fungi have provided new insights into how nuclear transport can be regulated in a cell cycle-specific manner. Nuclear transport occurs in a regulated manner through the aqueous channel of NPCs. These massive structures straddle the two membrane layers of the NE to provide a regulated conduit between the cytoplasm and nucleoplasm (Tran and Wente, 2006). During the transition from interphase to mitosis, transport of proteins to the nucleus has to be regulated. For instance, during interphase, microtubules form exclusively in the cytoplasm because tubulin is unable to transit the NPC. However, the mitotic spindle has to gain access to nuclear chromosomes in order to segregate them. How then does cytoplasmic tubulin gain access to nuclear chromosomes? In higher eukaryotes this problem is solved by NE breakdown during early mitosis. As the NE does not exist during such "open mitoses," there is no need to transport tubulin into the nucleus; the system is open. In the corn smut *U. maydis*, a similar process occurs whereby the genome is actively removed from the NE, allowing the mitotic spindle access to chromosomes in a form of open fungal mitosis (Straube et al., 2005). However, in many other fungi, including the model genetic organisms *S. cerevisiae*, *S. pombe*, *N. crassa*, *A. gossypii*, and *A. nidulans*, the NE persists during mitosis. These organisms therefore require a different mechanism to get tubulin into nuclei during mitosis. Little is currently known about these mechanisms. In *S. cerevisiae*, NPCs remain intact throughout mitosis, although protein-protein interactions between specific NPC proteins (Nups) change within the NPC structure (Makhnevych et al., 2003). This leads to changes in the transport properties of NPCs during mitosis (Makhnevych et al., 2003), but how this relates to tubulin transport is not known.

Work utilizing *A. nidulans* has uncovered a new type of mitosis-specific change in nuclear transport. In *A. nidulans*, tubulin released from cytoplasmic microtubules is unable to enter nuclei during interphase but can enter mitotic nuclei (Ovechkina et al., 2003). This demonstrates that a cell cycle-regulated mechanism for mitotic location of tubulin to nuclei, presumably involving cell cycle regulation of nuclear transport, exists in *A. nidulans*. Recent studies utilizing genetic analysis of NIMA-interacting genes have found this to be the case, but in a manner that was not anticipated.

The NIMA mitotic kinase interacts genetically with two components of the NPC, termed $SONA^{Gle2}$ and $SONB^{Nup98}$ ("SON" is an acronym for "suppressor of *nimA1*"), orthologues of highly conserved Nups found from yeasts to humans (De Souza et al., 2003; Wu et al., 1998). $SONB^{Nup98}$ is highly phosphorylated during mitosis in a NIMA- and Cdk1-dependent manner, indicating that it is mitotically regulated. Investigation of the location of $SONB^{Nup98}$ and $SONA^{Gle2}$ during mitosis found that they unexpectedly disassembled from NPCs at the G_2/M transition and returned back to NPCs during exit from mitosis (De Souza et al., 2004). These findings prompted studies of all the *A. nidulans* Nups, as well as other key nuclear transport proteins, revealing that NPCs partially disassembled during mitosis (De Souza et al., 2004; Osmani et al., 2006). Examination of the predicted location of these Nups within the NPC led to a new model for closed mitoses. In this model, Nups that are predicted to locate in the central transport channel, the so-called FG repeat Nups, as well as Nups located to the cytoplasmic and nucleoplasmic sides of the NPC structure, disperse from a core NPC structure specifically during mitosis (Fig. 5). The core NPC structure that remains at the NE during mitosis allows free diffusion of tubulin and

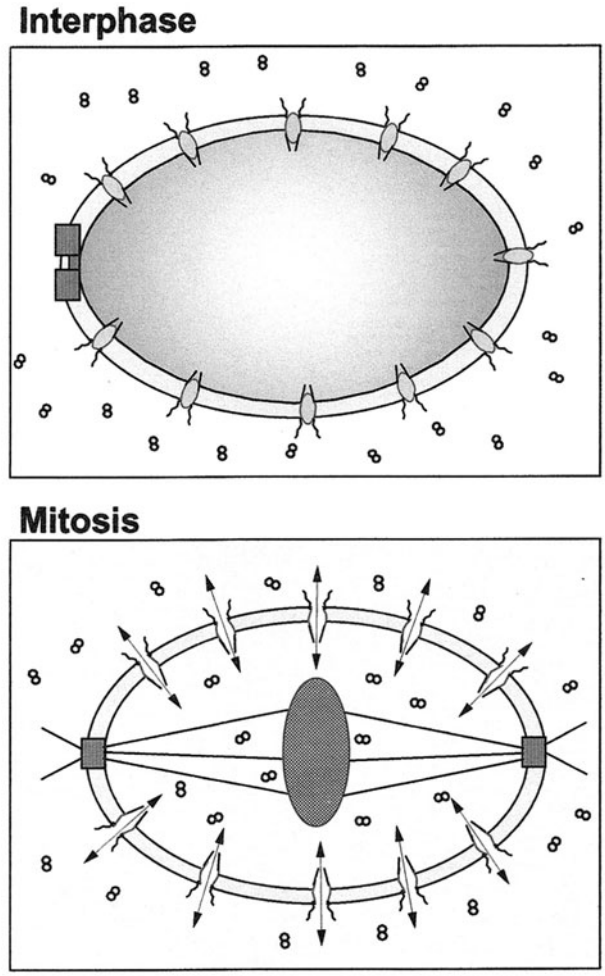

FIGURE 5 Schematic of interphase and mitotic *A. nidulans* nuclei. NPCs are embedded within the NE. During interphase, Nups occupy the central channel of NPCs, restricting diffusion. In mitosis, the NPCs partially disassemble such that the central channel is now open and the NE is permeable. This facilitates nuclear entry of tubulin, allowing spindle formation from the spindle pole bodies.

mitotic regulators into and out of nuclei (Osmani et al., 2006). In this partially open mode of mitosis, proteins localize within the cell depending on their respective binding affinities, similar to a mammalian open mitosis. Such mitosis-specific diffusion through the NE is consistent with prior studies showing that a reporter construct fused to a nuclear localization sequence is actively transported into nuclei during interphase but disperses from nuclei upon entry into mitosis (Suelmann et al., 1997). Therefore, opening of the NPC transport channel to allow diffusion between the cytoplasm and nucleoplasm provides the mechanism by which cytoplasmic tubulin gains access to nuclei, enabling formation of the nuclear mitotic spindle. During exit from mitosis, the core mitotic NPC structure acts as a prepore to which the dispersed Nups return and reestablish regulated nuclear transport and entry into G_1 (Fig. 5).

The genetic interaction between NIMA and Nups and the fact that these same Nups disperse from NPCs during mitosis suggest that NIMA plays a direct role to promote NPC disassembly. Several additional findings support this. As NIMA enters nuclei, it does so in a distinctive manner. Rather than seamlessly entering nuclei, as does tubulin, NIMA accumulates around the nuclear periphery during translocation, colocalizing with NPCs within the NE as described above (De Souza et al., 2004). Thus, NIMA is at the correct location at the right time to promote $SONB^{Nup98}$ phosphorylation and partial NPC disassembly. Importantly, as mentioned above, $SONB^{Nup98}$ is hyperphosphorylated during mitosis in a NIMA-dependent manner. Further, ectopic expression of NIMA promotes both the release of $SONB^{Nup98}$ from NPCs and the opening of the transport channel allowing diffusion of tubulin into nuclei (De Souza et al., 2004). Finally, dominant negative NIMA constructs accumulate at the nuclear periphery when overexpressed and delay entry into mitosis (De Souza et al., 2004). Therefore, NIMA is both necessary and sufficient to promote partial NPC disassembly and free diffusion into and out of nuclei to promote mitosis.

It is currently unclear whether other filamentous fungi, or other organisms in general, utilize partial NPC disassembly to regulate mitosis and what role NEKs may play if they do. However, similar to *A. nidulans*, some filamentous fungi that maintain an intact NE during mitosis have been demonstrated to display equilibration between the cytoplasmic and nuclear compartments specifically during mitosis. For instance, in the plant pathogens *Fusarium verticillioides* and *M. grisea*, the permeability of the NE changes dramatically during mitosis, allowing cytoplasmic proteins into mitotic nuclei (Bourett et al., 2002). In addition, cytoplasmic tubulin can enter the mitotic nuclei of *M. grisea* but not interphase nuclei (Czymmek et al., 2005). These mitotic changes in NE permeability may be mediated by partial NPC disassembly. In the case of *M. grisea*, this is particularly intriguing given that its NIMA orthologue is required for mitosis and can complement *A. nidulans nimA* mutants (Veneault-Fourrey et al., 2006).

Complete NPC disassembly has recently been demonstrated during mitosis in *U. maydis* yeast-like cells, which undergo a form of open mitosis (Theisen et al., 2008). Little is currently known about how NPC disassembly and reassembly are regulated during mammalian open mitosis, and these processes do not occur in model yeast systems. Therefore, model filamentous fungi such as *A. nidulans* and *U. maydis* provide unique opportunities to study mitotic regulation of the NPC.

THE LINKAGE OF CYTOKINESIS TO MITOTIC EXIT

Differential Regulation of Cytokinesis

As suggested by the fact that filamentous fungi often maintain many nuclei in a common cytoplasm, cytokinesis does not accompany every nuclear division. The process of cytokinesis during filamentous growth is generally achieved by the formation of a cross wall called a septum at a specific point in hyphae. In terms of how this relates to the cell cycle, this process is best understood in *A. nidulans*, for which genetic screens have been performed to isolate septation mutants (Harris et al., 1994; Morris, 1976b). During normal uninucleate conidial germination, septation does not occur in the first two or three cell cycles, resulting in either four or eight nuclei in the cytoplasm of a single germ tube (Fiddy and Trinci, 1976; Harris, 1997). However, following the third or fourth synchronous round of mitoses, a single septum asymmetrically forms, separating the resulting nuclei into two cellular compartments (Fig. 6A). Similar suppression of septation during the initial cell cycles also occurs during germination of *A. fumigatus* conidia (Momany and Taylor, 2000). Notably, when septation does occur, it is coordinated with the completion of mitosis, indicating that it is regulated by the cell cycle machinery. How septation is suppressed in early cell cycles and then induced in later rounds of mitosis is not well understood, although cell size

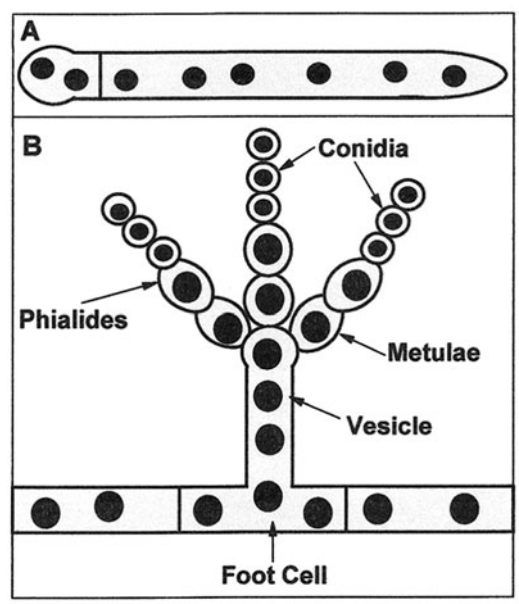

FIGURE 6 Septation is not always linked to nuclear division in *A. nidulans*. (A) A germling containing eight nuclei, which underwent its first asymmetric septation following the third mitosis. (B) Diagram of a conidiophore that has formed by growth of a multinucleate cell containing a stalk and vesicle. This cell originally formed by growth of a specialized aerial hypha termed the vesicle from the foot cell, in which multiple nuclear divisions occur without septation. Cytokinesis is linked to nuclear division during the formation of metulae and phialides as well as when uninucleate conidiospores form by budding from the phialide. Cytokinesis occurs through septation in hyphae, but by budding to form metulae, phialides and conidia.

TABLE 1 Cytokinesis and nuclear division in different *A. nidulans* cell types

Cell type	Cytokinesis and nuclear division
Early germling	Not linked
Hyphae	Linked
Foot cell	Specialized septa
Vesicle	Not linked
Phialides/metulae	Linked (pseudohypha-like budding)
Conidiospores	Linked (budding)

is thought to play an important role (Wolkow et al., 1996). Such suppression of septation in early cell cycles has been proposed to be a mechanism which subsequently accelerates polarized growth during the transition from germling growth to hyphal growth (Horio and Oakley, 2005). This is because the initial lack of septation generates a cytoplasm containing multiple nuclei increasing the biosynthetic capacity of a cell which is only growing in one direction at a single growth site. Consistent with this, the rate of growth of hyphal tips, driven by multiple nuclei, is some five times faster than the tip growth of small germlings (Horio and Oakley, 2005).

Further variations in the linkage of cytokinesis with nuclear divisions exist in other *A. nidulans* cell types (Fig. 6B and Table 1). The most dramatic variation occurs during *A. nidulans* asexual sporulation, in which cytokinesis is linked to every nuclear division, resulting in the formation of chains of uninucleate conidiospores in a budding-type process (Adams et al., 1998). Conidiospores are formed from a specialized cell called a phialide, which is part of a specialized structure called the conidiophore. The budding of conidiospores occurs by repeated mitotic nuclear divisions of the phialide nucleus such that one daughter nucleus remains in the phialide while the other enters the conidium and arrests in G_1 (Timberlake, 1990). Therefore, the phialide acts as a type of stem cell, which maintains its own identity and mitotic capacity while generating numerous other, mitotically inert, cells. It is likely that many of the genes that regulate hyphal septation are also involved in asexual budding, as mutants deficient in septation also abolish or greatly interfere with conidiation (Harris et al., 1994; Liu et al., 2003; Liu and Morris, 2000; McGuire et al., 2000). However, how these developmental changes in septation are integrated with the cell cycle machinery is not known.

Regulators of Septation

A septation initiation network has been described in the fission yeast *S. pombe* and is analogous to the mitotic exit network of budding yeast (Wolfe and Gould, 2005). These networks consist of a GTPase signaling cascade that coordinates septum formation and/or cytokinesis with the completion of each mitosis. Most components of an analogous pathway are present in filamentous fungi (Borkovich et al., 2004; Harris, 2008; Kim et al., 2006; Straube et al., 2005). Mutants in some of these components including *sepH*^{Cdc7}, *sidB*^{Sid2}, and *mobA*^{Mob1} compromise septation and conidiation in *A. nidulans* (Bruno et al., 2001; Kim et al., 2006). Interestingly, neither *sepH*^{Cdc7}, *sidB*^{Sid2}, nor *mobA*^{Mob1} is essential for colony formation or hyphal growth, while the fission yeast orthologues of these mutants are essential genes (Gould and Simanis, 1997). This likely reflects the essential nature of cytokinesis in uninucleate fission yeast in comparison to the multinucleate nature of filamentous fungi, such as *A. nidulans* (Kim et al., 2006).

Another cell cycle regulator which has been implicated in *A. nidulans* septation is *A. nidulans* Cdk1 (De Souza et al., 1999; Harris and Kraus, 1998; McGuire et al., 2000; Ye et al., 1999). High levels of Cdk1 activity induce early onset of septum formation in small cells and before the third round of mitosis (De Souza et al., 1999; Harris and Kraus, 1998). This has led to the proposal that a threshold level of Cdk1 activity is required to induce septation (Harris, 2001; Harris and Kraus, 1998). However, to date it has not been demonstrated that, during hyphal growth, Cdk1 activity is lower during the first and second rounds of mitosis than in the third, when the formation of the first septum is generally initiated. Therefore, it is still unclear how septation is suppressed until after the third mitosis in *A. nidulans* or how a single, asymmetric septum is formed (Fig. 6A). Cdk1 activity also interferes with cytokinesis in the basidiomycete *U. maydis*. During the yeast-like phase of its life cycle, *U. maydis* undergoes budding which differs from that of ascomycetous yeasts in that two distinct septa, rather than one, are formed in each budding event. Genetic manipulations that increase Cdk1 activity interfere with this budding process, resulting in chains of cells separated by septa (Sgarlata and Perez-Martin, 2005a, 2005b). Therefore, Cdk activity is important for the regulation of cytokinesis and septation in both the ascomycete and basidiomycete filamentous fungi.

Another interesting observation with regards to septation in *A. nidulans* is that DNA damage can inhibit septum formation (Harris and Kraus, 1998). Further, some mutants defective in septation are thought to indirectly prevent septation by causing genotoxic damage. In such mutants, the septation defects are suppressed when combined with DNA damage checkpoint-deficient mutants (Harris and Kraus, 1998; Wolkow et al., 1996). Therefore, filamentous fungi have a mechanism to prevent septation if DNA damage has occurred. This may help maintain multiple copies of the genome in one cellular compartment, which increases the chance of having at least one functional copy of an essential gene in each cell. For example, if DNA damage knocks out an essential gene in only one G_1 nucleus of a cell containing several nuclei, preventing septation ensures that no cells are generated which contain only a null allele of an essential gene. Potential mechanisms such as these suggest that filamentous fungi have evolved specialized checkpoint mechanisms regulating septation.

SYNCHRONY OF CELL CYCLE PROGRESSION IN MULTINUCLEATED CELLS

One of the intriguing aspects of syncytial, multinucleate fungi is how they coordinate cell cycle events such as mitosis between nuclei in a common cytoplasm. This can vary among different organisms, which utilize either synchronous, parasynchronous, or asynchronous modes of nuclear division (Gladfelter, 2006). For example, *A. nidulans* hyphae use a parasynchronous mode, in which mitosis occurs as a wave generally beginning at the hyphal tip and then spreading along the rest of the cell. In contrast, *N. crassa* and *A. gossypii* undergo asynchronous nuclear divisions, and therefore mitosis is "nuclear autonomous" in these organisms (Gladfelter et al., 2006; Serna and Stadler, 1978). As we have discussed above, the biochemical activities that regulate mitotic entry are conserved in different

filamentous fungi. How different organisms utilize these same activities but display such dramatically different modes of timing nuclear division has long been a mystery, but it is likely that cellular compartmentalization of mitotic regulators is important. As the mitotic apparatus is set up within the individual nuclei of a syncytium, the key may be in how these mitotic regulators gain access to nuclei. As we have described, the NE is permeable during *A. nidulans* mitosis, due to the partial disassembly of NPCs. In other organisms, NPCs may remain intact, as occurs in budding yeast (Makhnevych et al., 2003). In these organisms, modification of nuclear transport pathways is likely important for nuclear entry of mitotic regulators. One possibility is that modification of nuclear transport pathways can be modulated in a nuclear autonomous manner, whereas partial NPC disassembly, which is likely initiated in the cytoplasm, cannot. Given that partial NPC disassembly is unlikely to occur in a nuclear autonomous manner, it is not likely to be utilized by organisms undergoing asynchronous mitoses within a syncytium. Future studies will no doubt shed light on these and other possible mechanisms, such as nuclear autonomous activation of the APC and protein degradation, for temporally regulating asynchronous mitosis within syncytia.

Interestingly, while mitosis within *A. nidulans* syncytia is parasynchronous under optimal conditions, this synchrony breaks down when cells are grown on nutrient-poor media (Clutterbuck, 1970; Rosenberger and Kessel, 1967). Therefore, *A. nidulans* can change its mode of mitosis in response to environmental conditions that affect growth rate. If environmental conditions dictate slow growth, it may be more practical to divide one nucleus at a time, as this requires less energy than synchronously dividing multiple nuclei. It remains to be seen if such asynchronous *A. nidulans* mitoses are regulated by partial NPC disassembly or if another mechanism is utilized.

G_2 DNA DAMAGE AND SLOWED S-PHASE CHECKPOINTS

Eukaryotic cells utilize safeguard mechanisms to prevent cell cycle progression if DNA is not completely replicated or if DNA is damaged. Such mechanisms have been defined as cell cycle checkpoints, which, by stopping the cell cycle, allow cells time to complete replication or repair DNA damage before entering mitosis. Given the critical nature of such checkpoints, it is not surprising that mutations in many key cell cycle checkpoint genes have been identified in human genetic disorders, such as ataxia-telangiectasia and Nijmegen breakage syndrome (Lavin, 2007). Cell cycle checkpoints are therefore a crucial part of the DNA damage response and are integrated with detection of DNA damage and the activation of DNA repair pathways. Below is reviewed what is known about the cell cycle checkpoint branch of the DNA damage response in filamentous fungi. Readers are also referred to reviews on other aspects of the DNA damage response (Goldman et al., 2002; Goldman and Kafer, 2004).

As outlined above, activation of the Cdk1 kinase via dephosphorylation of its tyrosine residue is required for mitotic entry (Fig. 2). Most eukaryotic cells target this Cdk1-activating step to prevent mitotic entry if DNA is damaged or incompletely replicated (Jin et al., 1996; Rhind et al., 1997; Ye et al., 1997). A notable exception to this is the budding yeast *S. cerevisiae* (Amon et al., 1992; Sorger and Murray, 1992), which arrests at the metaphase-to-anaphase transition by inhibiting sister chromatid separation in response to DNA damage (Cohen-Fix and Koshland, 1997). This likely reflects the fact that this organism does not have a G_2 phase of the cell cycle, since spindle formation begins as DNA is replicated in S phase. However, in filamentous fungi and other organisms mutations that inhibit the tyrosine phosphorylation of Cdk1 cause defects in the G_2 DNA damage checkpoint. For example, in *A. nidulans*, strains in which the phosphorylatable threonine and tyrosine residues within Cdk1 have been mutated to nonphosphorylatable alanine and phenylalanine residues (Cdk1 AF mutant) fail to arrest in G_2 in response to DNA damage (Ye et al., 1997). The failure of the G_2 DNA damage checkpoint in such cells results in enhanced sensitivity to DNA-damaging agents. Mutations in the *ankA*^{wee1} kinase responsible for tyrosine phosphorylation of Cdk1 (Fig. 2) also cause checkpoint defects and DNA damage sensitivity (Ye et al., 1997).

The inability to phosphorylate Cdk1 on threonine and tyrosine also results in loss of the prolonged S-phase checkpoint that prevents mitotic entry if DNA replication is not completed (Ye et al., 1996). Therefore, *A. nidulans* Cdk1 AF and Δ*ankA*^{wee1} mutants inappropriately enter mitosis even if DNA replication is not completed. This results in sensitivity to low concentrations of the ribonucleotide reductase inhibitor HU, which interferes with DNA replication. In *U. maydis*, preventing tyrosine phosphorylation of Cdk1 also results in sensitivity to HU, suggesting that a similar prolonged S-phase checkpoint exists in Basidiomycetes (Sgarlata and Perez-Martin, 2005a).

In *A. nidulans*, completely inhibiting DNA replication by using high concentrations of HU revealed a second checkpoint that monitors DNA replication. In these studies, Cdk1 AF mutants inappropriately entered mitosis if replication was slowed but did not enter mitosis if replication was completely inhibited. Moreover, Cdk1 AF mutants displayed marked sensitivity to low concentrations of HU but no enhanced sensitivity to transient exposure to concentrations of HU that completely block DNA replication. This revealed a second checkpoint, termed the S-phase arrest checkpoint, which responds to a complete block in DNA replication. The S-phase arrest checkpoint can prevent mitosis even in the absence of Cdk1 tyrosine phosphorylation. Interestingly, loss of BIME^{APC1} function together with lack of Cdk1 tyrosine phosphorylation was found to negate the S-phase arrest checkpoint (Ye et al., 1996). This is likely because the loss of BIME^{APC1} function, together with activation of Cdk1, results in the accumulation of the active phosphorylated form of NIMA. Therefore, in these cells, Cdk1 and NIMA are both active, and this is sufficient to allow mitotic entry even without DNA replication. These studies provided further evidence of a role for the APC during interphase.

While Cdk1 tyrosine phosphorylation is crucial in preventing inappropriate mitotic entry in response to genomic defects, less is known about how cells know when to engage such checkpoints. To achieve this, stalled replication forks or damaged DNA must first be detected by the cell, and then pathways leading to Cdk1 tyrosine phosphorylation must be activated. To identify upstream regulators of Cdk1 tyrosine phosphorylation, the library of DNA damage-sensitive mutants available for *A. nidulans* was screened for sensitivity to low concentrations of HU. The rationale was that as Cdk1 tyrosine phosphorylation is required for both the G_2 DNA damage and prolonged S-phase checkpoints, regulators of Cdk1 tyrosine phosphorylation should be sensitive to both DNA damage and a slowed S phase. Of these

mutants, only the four mutant alleles of *uvsB* and two of *uvsD* displayed sensitivity to HU, in addition to their defined DNA damage sensitivities. These genes were subsequently cloned by complementation of their HU sensitivity, and sequencing indicated that UVSB was closely related to fission yeast Rad3, while UVSD displayed low homology to Rad26 (De Souza et al., 1999; Hofmann and Harris, 2000). Both *uvsB* and *uvsD* mutants failed to arrest in G_2 in response to DNA damage, indicating that they did indeed function in the G_2 DNA damage checkpoint, similar to their *S. pombe* orthologues (al-Khodairy et al., 1994; al-Khodairy and Carr, 1992). However, while Rad3 was known to be a member of the ATM-related (ATR) protein kinase family, orthologues of Rad26 were not known. Subsequently, the human UVSD/Rad26 orthologue ATR-interacting protein (ATRIP) was identified and shown to be phosphorylated by ATR as part of the checkpoint response (Cortez et al., 2001). Recently, *N. crassa uvs-3* was shown to be a member of the UVSD/Rad26/ATRIP family (Kazama et al., 2008). Further, *uvsD* can partially complement the HU sensitivity of *uvs-3* mutants, indicating functional conservation of this checkpoint in another filamentous fungus.

Another key player in cell cycle checkpoints is the ATM checkpoint kinase. ATM is related to the ATR checkpoint kinase but is thought to be more specific for the response to DNA double-strand breaks, whereas ATR responds to a wider range of genotoxic damage. Reverse genetics have been used to identify and delete the *A. nidulans* ATM orthologue, $atmA^{ATM}$, demonstrating that it is required for the proper checkpoint response to DNA double-strand breaks. Recently it has been shown that strains lacking both $uvsB^{ATR}$ and $atmA^{ATM}$ are not viable (Malavazi et al., 2008), although interestingly this is not the case in budding yeast (Morrow et al., 1995). More surprisingly, however, $atmA^{ATM}$ nulls also display defects in polarized hyphal growth (Malavazi et al., 2006). Findings such as these suggest that the cell cycle checkpoint machinery may be integrated with hyphal morphogenesis, although the mechanisms involved remain to be elucidated.

The MRN (Mre11, Rad50, and Nbs1) complex cooperates with ATM and ATR to generate signals that activate cell cycle checkpoints (D'Amours and Jackson, 2002; Lavin, 2007; Zhang et al., 2006). Consistent with this, mutants in the *A. nidulans* orthologues of these genes have defects in cell cycle checkpoints (Bruschi et al., 2001; Malavazi et al., 2005; Semighini et al., 2003). Of these genes, $scaA^{Nbs1}$ was identified in a screen for mutants sensitive to the DNA topoisomerase I inhibitor camptothecin, while $mreA^{Mre11}$ was subsequently identified in a two-hybrid screen using a $scaA^{Nbs1}$ bait (Bruschi et al., 2001; Semighini et al., 2003). Intriguingly, $sldI^{Rad50}$ was identified in a screen for genes which are synthetically lethal with the loss of function of the microtubule motor dynein (Malavazi et al., 2005).

Another interesting connection between the cell cycle and the DNA damage response comes from the finding that a mutation in $sonB^{Nup98}$, which encodes an NPC protein, causes DNA damage sensitivity (De Souza et al., 2006). Interestingly, this mutation is conditional in that mutants are not DNA damage sensitive at 32°C but display marked sensitivity at 42°C. At the same elevated temperature, this $sonB^{Nup98}$ mutation has also been shown to suppress a *nimA1* G_2 arrest (De Souza et al., 2003), and this was the way this mutation was first identified. However, despite this role in the G_2/M transition, $sonB^{Nup98}$ mutants were found to have an intact G_2 DNA damage checkpoint. Rather, the role for $sonB^{Nup98}$ in the DNA damage response may involve the MRN complex as the $sonB^{Nup98}$ mutant is synthetically lethal with $scaA^{Nbs1}$ mutants at 42°C but not at 32°C. This suggests that $SONB^{NUP98}$ plays a novel role in the DNA damage response and reveals a potential link between the cell cycle, NPCs, and the DNA damage response.

The Chk1 and Chk2 kinases are components of the signaling pathways that activate cell cycle checkpoints in response to the detection of DNA damage (Chen and Poon, 2008). The corresponding genes have recently been deleted in *A. nidulans*, and the roles of these kinases in the DNA damage response have begun to be characterized (Malavazi et al., 2008). Intriguingly, the *N. crassa* Chk2 orthologue, *prd-4*, was identified as a gene involved in the circadian system, revealing an interesting link between this clock system and the cell cycle (Pregueiro et al., 2006).

One interesting question with regard to checkpoint regulation within a fungal syncytium is whether a defect within a single nucleus can activate cell cycle checkpoints, such that all nuclei arrest. For example, if DNA damage occurs in only one nucleus, do other nuclei within the same cell arrest in G_2? Technology now exists to address such questions, using lasers to specifically elicit damage to a single nucleus within a syncytium.

VARIATIONS ON THE MODE OF MITOSIS WITHIN FUNGI

Filamentous fungi display stunning diversity in how the genome is physically segregated by the mitotic apparatus. For example, while many fungi maintain an NE around the spindle in a closed mitosis, basidiomycetes such as *U. maydis* strip the mitotic apparatus from the NE. Morphological differences such as these were readily identifiable in early studies of fungal mitosis using light or electron microscopy and have been elegantly described in the literature (Aist and Morris, 1999; Heath, 1980; O'Donnell and McLaughlin, 1984; Robinow and Caten, 1969). However, more-recently identified variations, such as the partial NPC disassembly during mitosis in *A. nidulans*, became apparent only by use of more modern tools, such as live cell imaging of fluorescently tagged proteins. It is likely that other mitotic variations await discovery. It is clear, however, that referring to mitosis as either open or closed has its limitations. For example, molecules at least a large as 250 kDa can defuse in and out of nuclei during *A. nidulans* mitosis (Osmani et al., 2006). Therefore, although the NE is intact, it is a partially open system.

Many of the mitotic variations described in filamentous fungi are variations on how the NE is segregated to generate two daughter nuclei during closed mitosis (Heath, 1980). The NE acts as a barrier between the nucleus and cytoplasm and as such keeps kinetochores in the nucleus away from cytoplasmic microtubules. In organisms undergoing closed mitosis, the mitotic spindle is formed by microtubules nucleated from the nucleoplasmic face of the spindle pole body. One advantage of undergoing closed mitosis is that it restricts the attachment of spindle microtubules to only those kinetochores within that nucleus. This is presumably highly advantageous in filamentous fungi that contain multiple nuclei within a syncytium, as it prevents internuclear spindles (see De Souza and Osmani, 2007, for a discussion of this topic). Therefore, multinucleate fungi tend to maintain their NE intact until at least metaphase, when all microtubule attachments have been made. Subsequent to this, however, there is

significant variation in how segregation of DNA and the NE are coordinated (Heath, 1980).

How the NE undergoes restriction and fission to generate two daughter nuclei during the closed mitoses of yeasts and fungi is still a complete mystery. Little is known about how NE fission is regulated or what the motor proteins involved are. Clearly NE fission has to occur after segregation of DNA on the mitotic spindle. In *A. nidulans*, there are many examples of mitotic mutants that enter mitosis but fail to undergo NE fission, generating polyploid nuclei surrounded by a single contiguous NE (Davies et al., 2004; De Souza et al., 1999; May et al., 1992; Pitt et al., 2004). In some cases, DNA appears to segregate, but examination of the NE reveals that it still connects the DNA masses. Therefore, DNA segregation can be uncoupled from NE fission, suggesting that although normally coordinated, these are distinct processes. Further, some mutants that fail to complete NE fission undergo subsequent rounds of DNA replication (De Souza et al., 1999; May et al., 1992; Pitt et al., 2004). One possibility is that this allows cells to increase their biosynthetic capacity, even without nuclear division. As long as such cells also have other nuclei which have normal haploid ploidy, they should be able to continue growth and eventually undergo asexual development.

Perhaps even more fascinating than the described mitotic variations among species are the mitotic variations which occur in different cell types within one organism. In fact, given that septation is mitotically linked but does not occur in every mitosis or cell type, all filamentous fungi display some mitotic variations during their life cycle. In some cases, specialized cellular structures are made to facilitate these mitotic variations, for example, the *A. nidulans* conidiophore. Another intriguing example of a specialized cell structure for mitosis is the clamp that is formed during the dikaryotic stage of many basidiomycetes (Iwasa et al., 1998; Scherer et al., 2006). As such dikaryons contain two genetically distinct nuclei, it is important that following mitosis the two new cellular compartments inherit one of each type of nucleus. The clamp helps cells achieve this by providing a small branch in which one nucleus undergoes mitosis while, at the same time, the other nucleus undergoes mitosis in the germ tube (Fig. 7). The formation of specialized septa then allows the successful generation of dikaryotic cells. Clearly, the spatial regulation of the cytoskeleton and septum formation by mitotic regulators is also important to facilitate this fascinating mode of mitosis. Additionally, it has been suggested that although basidiomycetes, such as *U. maydis*, undergo open mitosis in their yeast phase of growth, this is unlikely to occur during the dikaryotic phase (De Souza and Osmani, 2007). This is because such open mitoses would likely allow interference between the spindle apparatus of the different nuclei synchronously undergoing mitosis within the same cell. This leads to the interesting possibility that open and closed mitoses might occur in fungi in a developmentally regulated manner, as is known to occur in *Physarum polycephalum* (Solnica-Krezel et al., 1991; Tanaka, 1973).

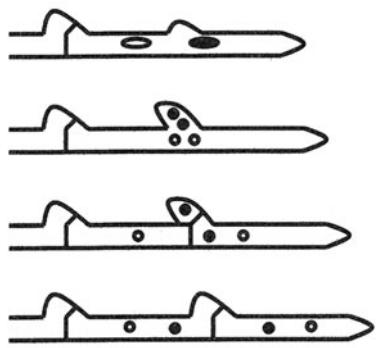

FIGURE 7 Schematic showing how development of a clamp and specialized septa help maintain the dikaryotic state of basidiomycete hyphae (adapted from Iwasa et al., 1998).

DEVELOPMENTAL CONTROL OF CELL CYCLE PROGRESSION IN MULTICELLULAR FUNGI

Many filamentous fungi have life cycles in which developmentally differentiated cell types form. Such changes are a prerequisite for sexual or asexual reproduction and are often involved in host cell invasion by pathogenic fungi (Gow et al., 2002; Steinberg, 2007). These developmental changes are initiated by environmental factors triggering different developmental and transcriptional programs (Adams et al., 1998; Borkovich et al., 2004; Steinberg, 2007). In *A. nidulans*, many of the transcriptional factors regulating asexual conidiophore development have been characterized (Adams et al., 1998). For example, mutation of the *brlA* transcription factor prevents asexual conidiophore formation, while inappropriate expression in vegetative cells can induce conidiospore formation when it normally would not occur (Adams et al., 1988). Such ectopic expression of *brlA* has been shown to induce the mRNA expression and increase activity of the Cdk1/cyclin B and NIMA mitotic kinases (Ye et al., 1999). This suggests that levels of these kinases are increased during asexual development, in a *brlA*-dependent manner. Consistent with this, Cdk1/cyclin B and NIMA kinase activities increase sixfold during conidiophore development (Ye et al., 1999). This provides strong evidence that components of the mitotic machinery are developmentally regulated. Interestingly, during asexual conidiophore formation, a second *brlA*-inducible cyclin, Pcl2, is involved in Cdk1 regulation in addition to $NIME^{CyclinB}$ (Schier and Fischer, 2002). Together, these studies indicate that during development, Cdk1 activity is increased and regulated by multiple cyclin binding partners. However, as Cdk1 AF mutants display many developmental anomalies such as septation in the stalk and vesicle (Ye et al., 1999), it is clear that this activity must still be correctly regulated by tyrosine phosphorylation.

Other Cdks, such as members of the Cdk5/Pho85 family, are not directly involved in cell cycle regulation but have roles in developmental regulation. In *A. nidulans*, this includes the PHOA and PHOB Pho85-like Cdks, which together with their PHO80 cyclin partner, are involved in the developmental switch between asexual and sexual reproduction in response to environmental stimuli (Bussink and Osmani, 1998; Dou et al., 2003; Wu et al., 2004). Further, the *U. maydis* Cdk5 together with its cyclin partner, Pcl12, has important roles in regulating morphogenesis and pathogenesis (Castillo-Lluva et al., 2007; Flor-Parra et al., 2007). Therefore, filamentous fungi utilize a more sophisticated Cdk-cyclin machinery to regulate developmental decisions that are not required in unicellular yeasts.

CELL CYCLE CONTROL AND PATHOGENESIS

Pathogenic fungi often undergo morphological changes during host invasion (Gow et al., 2002). Such morphological changes can entail switching between budding and filamentous modes of growth. In large part, this is achieved by

regulating septation and cytokinesis, which, as we have discussed above, are under cell cycle control. For example, at 25°C the nonpathogenic form of the dimorphic fungal pathogen *Penicillium marneffei* undergoes filamentous vegetative growth, in which cells are generally multinucleate. However, this organism is pathogenic at 37°C, where conidia germinate and form hyphae, which in turn produce arthroconidia. Arthroconidia are uninucleate cells that arise because nuclear division and septation have become coupled. Breakdown of the material between the double septa in these cells results in the formation of yeast-like cells that divide by fission (Canovas and Andrianopoulos, 2007). Clearly then, the regulation of cytokinesis and mitosis is important for the pathogenicity of this and other fungi.

Links between cell cycle regulators and pathogenicity have also been uncovered in the maize smut fungus, *U. maydis*, as recently reviewed (Perez-Martin et al., 2006). During the infection process, haploid yeast-like sporidia of opposite mating types arrest in G_2 and fuse to form the pathogenic filamentous dikaryon (Garcia-Muse et al., 2003; see also chapter 34). This G_2 cell cycle arrest is therefore important for pathogenicity (Perez-Martin et al., 2006). Further, links between Cdks and pathogenesis have been revealed by preventing Cdk1 tyrosine phosphorylation, which stops the initiation of pathogenic development, perhaps due to a failure of cells to arrest in G_2 (Sgarlata and Perez-Martin, 2005a). Notably, interfering with either *U. maydis* Cdk1 phosphorylation or levels of B-type cyclins at later stages of plant infection also decreases virulence (Garcia-Muse et al., 2004; Sgarlata and Perez-Martin, 2005a). In addition, specific cyclins and other cell cycle regulators are important for regulating the morphogenic changes in the opportunistic human pathogen *C. albicans*, although whether or not this is important for virulence remains controversial (reviewed by Berman, 2006, and Whiteway and Bachewich, 2007).

Infection caused by the rice blast *M. grisea* has been demonstrated to be tightly linked to the cell cycle (Veneault-Fourrey et al., 2006). In this organism, a mitosis occurs in which one daughter nucleus migrates into an infection structure called the appressorium. Inhibiting the cell cycle by drug treatment before this mitosis greatly affects appressorium development, whereas drug treatment after this mitosis has little effect. To investigate this further, Talbot and colleagues turned to molecular genetics and generated a version of the *M. grisea nimA* orthologue containing the equivalent mutation, which causes a G_2 arrest at the restrictive temperature in *A. nidulans*. This $MgnimA^{E37G}$ strain was defective in appressorium formation at a nonpermissive temperature, providing further evidence that mitosis is required for this process and subsequent plant infection by *M. grisea* (Veneault-Fourrey et al., 2006). Therefore, cell cycle events such as mitosis may be linked to pathogenicity if these events are a prerequisite for formation of an infectious structure such as the *M. grisea* appressorium. Interestingly, however, this is not the case in the closely related plant pathogen *Colletotrichum gloeosporioides*, in which appressorium formation is not dependent on mitosis (Nesher et al., 2008).

SUMMARY

Filamentous fungi provide rich and varied experimental opportunities to further our understanding of mitosis and its regulation. Until relatively recently, *A. nidulans* has been the workhorse of the filamentous fungi for studies of mitosis. This situation is largely a result of the pioneering work of Ron Morris, whose genetic screens laid the foundation for the isolation of many cell cycle-specific genes. Clearly, there is much still to be understood about mitotic regulation in humans as well as in filamentous fungi. Equally clear are the vast opportunities provided by other fungi whose varied biology provides unique experimental advantages for the study of various aspects of mitosis. As outlined in this review, areas of particular interest are how the cell cycle is coordinated with morphogenesis and/or pathogenicity and the molecular basis of synchronous versus asynchronous cell cycles within syncytia. *A. gossypii* and *N. crassa* provide unique model systems in which to define how such asynchronous mitoses are regulated within a common cytoplasm. Other areas primed for further insights are the dramatic structural changes that occur in nuclei, in addition to the intensively studied processes of chromosome condensation and spindle formation. Many of these changes, such as NPC disassembly, do not occur in model yeasts, and therefore model fungi such as *A. nidulans* and *U. maydis* provide excellent systems with which to study these processes. With the current availability of high-quality genome data and recent improvements in gene targeting and proteomic technologies for many filamentous fungi, we look forward to the next cycle of mitotic insights from these important and fascinating organisms.

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7

Meiosis

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INTRODUCTION

In fungi that have a sexual cycle, meiosis is the process by which chromosomes recombine and haploid nuclei are generated. Meiosis consists of one round of DNA replication followed by two rounds of division (Fig. 1). The first division, termed reductional, separates homologs (thus reducing the ploidy of nuclei from diploid to haploid). The second division, termed equational, is like mitosis in that sister centromeres separate. The two divisions required by meiosis pose a number of problems that cells need to address. First, homologous chromosomes (homologs) need to find one another (pair). Second, each pair of homologs (each bivalent) must remain associated until the first division, and recombination plays an important role in this association between homologs. Third, the associations between sister chromatids (cohesion) must be modulated along the length of the chromosomes in specific ways through the two meiotic divisions. Filamentous fungi such as *Sordaria macrospora*, *Neurospora crassa*, and *Coprinus cinereus* (reclassified as *Coprinopsis cinerea* [Redhead et al., 2001]) have been and continue to be valuable organisms for the elucidation of meiotic processes, as reviewed previously (Pukkila, 1994; Zickler, 2006).

This chapter discusses the hallmarks of meiosis, crossover (chiasma) distribution, genes necessary for meiosis, and the transcriptional program of meiosis, with a focus on recent studies. A recent review, "*Neurospora* as a model fungus for studies in cytogenetics and sexual biology at Stanford" (Raju, 2009), is highly recommended as a complement to this chapter, which does not encompass the elegant studies described therein.

Pairing and Synapsis

Filamentous fungi display the interesting characteristic of maintaining haploid nuclei throughout mycelial development until nuclear fusion (karyogamy), which begins meiosis. Therefore, by definition, homologs cannot associate prior to karyogamy, and so any pairing must occur de novo and cannot be due to any premeiotic associations. Once karyogamy has occurred, nuclei proceed into the leptotene stage of meiosis. By the end of leptotene, homologous chromosomes have paired. In most organisms a proteinaceous structure, the synaptonemal complex (SC), forms between paired homologs (reviewed by Henderson and Keeney, 2005, and Page and Hawley, 2004), and formation of the SC, termed synapsis, begins to occur during zygotene. Pachytene is, by definition, the stage at which chromosomes are fully synapsed (Fig. 2).

While complete SC is found in most organisms, there are notable exceptions, including the fungi *Schizosaccharomyces pombe*, *Aspergillus nidulans*, and *Ustilago maydis* (Egelmitani et al., 1982; Fletcher, 1981; Olson et al., 1978; discussed by Zickler, 2006), suggesting that the structure is not absolutely required for meiosis and raising questions about its purpose (Page and Hawley, 2004). The SC is formed by the initial deposition of lateral/axial elements along chromosome axes, followed by the addition of transverse filaments perpendicular to the homolog axes, and central elements. Chromosomes condense during SC formation, and SC proteins play a role in this compaction (Page and Hawley, 2004). On exit from pachytene, the SC disassembles and nuclei enter diplotene. Synapsis differs from pairing in that it does not absolutely require homology and can occur between nonhomologous chromosomes (Page and Hawley, 2004).

Recombination and Crossover Formation

The formation of crossovers is essential for meiosis in most organisms that have been examined. For a pair of homologs to biorient on the metaphase I spindle and to separate in meiosis I, a stable association must form between them, and crossovers, in combination with cohesion between sister chromatids, form this association (Fig. 3).

Recombination begins with the formation of a DNA double-strand break (DSB). The break is then resected in the 5′-to-3′ direction on both strands, leaving single-stranded tails. One of these ends then invades the DNA duplex of a homologous chromatid, with an extreme bias (in meiosis) towards a nonsister (i.e., a chromatid of the homolog). Strand invasion leads to the formation of a D-loop. In crossover

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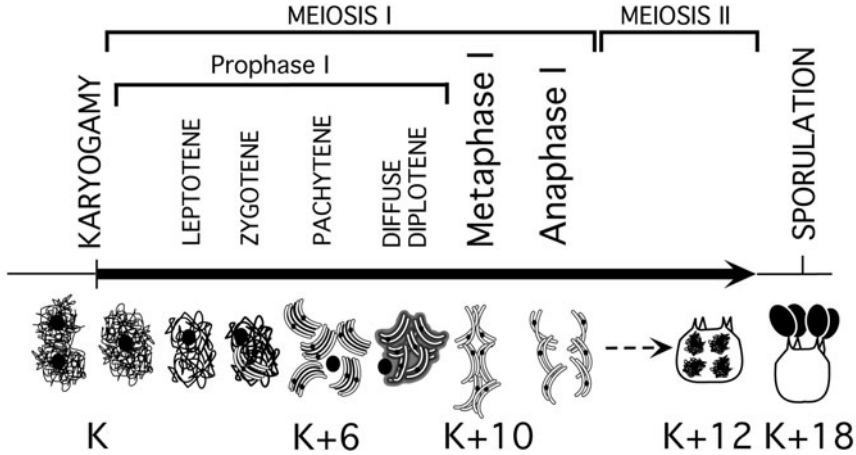

FIGURE 1 Progression and timing of meiosis in *C. cinereus*. Meiosis in *C. cinereus* is synchronous and begins directly after karyogamy (K). Homologs pair, condense, and synapse, and all meiotic cells are in pachytene 6 h postkaryogamy. After a further 2 to 3 h, homologs separate in the first meiotic division. Twelve hours after karyogamy, the second division has occurred, resulting in four meiotic products. Six hours subsequently, nuclei have migrated into basidiopores. Reproduced from the *EMBO Journal* (Celerin et al., 2000), with the permission of the publisher.

formation, the displaced loop anneals to the other single-stranded, resected end formed by the original DSB.

In *Saccharomyces cerevisiae*, DNA synthesis and ligation occur, forming a double Holliday junction that resolves as a crossover (Allers and Lichten, 2001); this is illustrated in Fig. 3. However, in *S. pombe*, a single Holliday junction intermediate generates a crossover (Cromie et al., 2006). Because this feature has been studied in only these two organisms, it is not known whether a single or double Holliday junction is the more common intermediate. In *S. cerevisiae*, the double Holliday junction then resolves to form a reciprocal crossover, which, in concert with cohesion, prevents precocious homolog separation (Fig. 3). Recombination, gene conversion, and postmeiotic segregation are thoroughly discussed elsewhere in this volume (chapter 8).

Cohesion and Divisions

The formation of a crossover alone is not sufficient to keep homologs together until the first meiotic division; sister chromatid cohesion is also required. Cohesion is mediated by the cohesin complex, which consists of four protein subunits that form a ring-like structure (see Watrin and Peters, 2006, for diagram). One popular model is that the ring structure "embraces" the two sister chromatids (Haering et al., 2008); this is depicted in Fig. 3. The presence of a crossover means that cohesion between original sister chromatids forces homologs to stay together (Fig. 3J). The separation of homologs and then of sister centromeres in two separate meiotic divisions necessitates the stepwise release of cohesin. At anaphase I, cohesin is released from the arms of the chromosomes but remains at the centromere (Revenkova and Jessberger, 2005). This allows homologs to separate. At the second division, centromeric cohesin is lost and sister centromeres separate. The meiotic cohesin complex contains a meiosis-specific subunit, Rec8. Rec8 associates with other proteins at the centromere that protect it from degradation at anaphase I (Wang and Dai, 2005).

The two meiotic divisions produce four haploid nuclei, which are sometimes followed by a postmeiosis mitosis, either before (as in *N. crassa*) or after (as in *C. cinereus*) spore formation. In *N. crassa*, eight ascospores are produced, in which each chromosome is derived from one of the eight DNA strands in the corresponding original pair of homologs (Raju, 1980). *C. cinereus* produces four basidiospores, each containing two nuclei (Kues, 2000).

Filamentous Fungi and Meiosis

Filamentous fungi have several advantages for the study of meiosis. First, the meiotic program and chromosome behavior in filamentous fungi are similar to those of more complex organisms. The genetic and cytological tractability of fungal systems allows genes to be well characterized, shedding light on the functions of conserved proteins. Second, unique aspects of development in filamentous fungi provide ideal conditions for analysis of certain meiotic events. Short fungal life cycles and the abundant production of meiotic cells facilitate analysis. For example, in *C. cinereus*, dikaryotic mycelia produce mushrooms within 1 to 2 weeks under the right conditions, and each mushroom contains approximately 10 million synchronous meiotic cells arrayed on the surface of hundreds of gills (Pukkila et al., 1984). The four products of meiosis (tetrads) can be recovered for analysis, and the linear arrangement of ascospores in some species (e.g., *N. crassa* [Raju, 1980]) is a direct consequence of the meiotic divisions, allowing further discernment of meiotic events. Third, the chromosomes of some filamentous fungi are particularly accessible for meiotic study, within intact cells or by surface spreads of meiotic nuclei (for examples, see Raju and Lu, 1970; Pukkila and Lu, 1985; Raju, 1992; Seitz et al., 1996; and Zickler, 2006).

The burgeoning publication of fungal genomes and increasing ease of transcriptomic and proteomic studies will facilitate identification of meiotic orthologs in fungi not traditionally associated with meiotic research. The wealth of data will allow us to build hypotheses regarding the conservation of meiosis as a process and that of the components

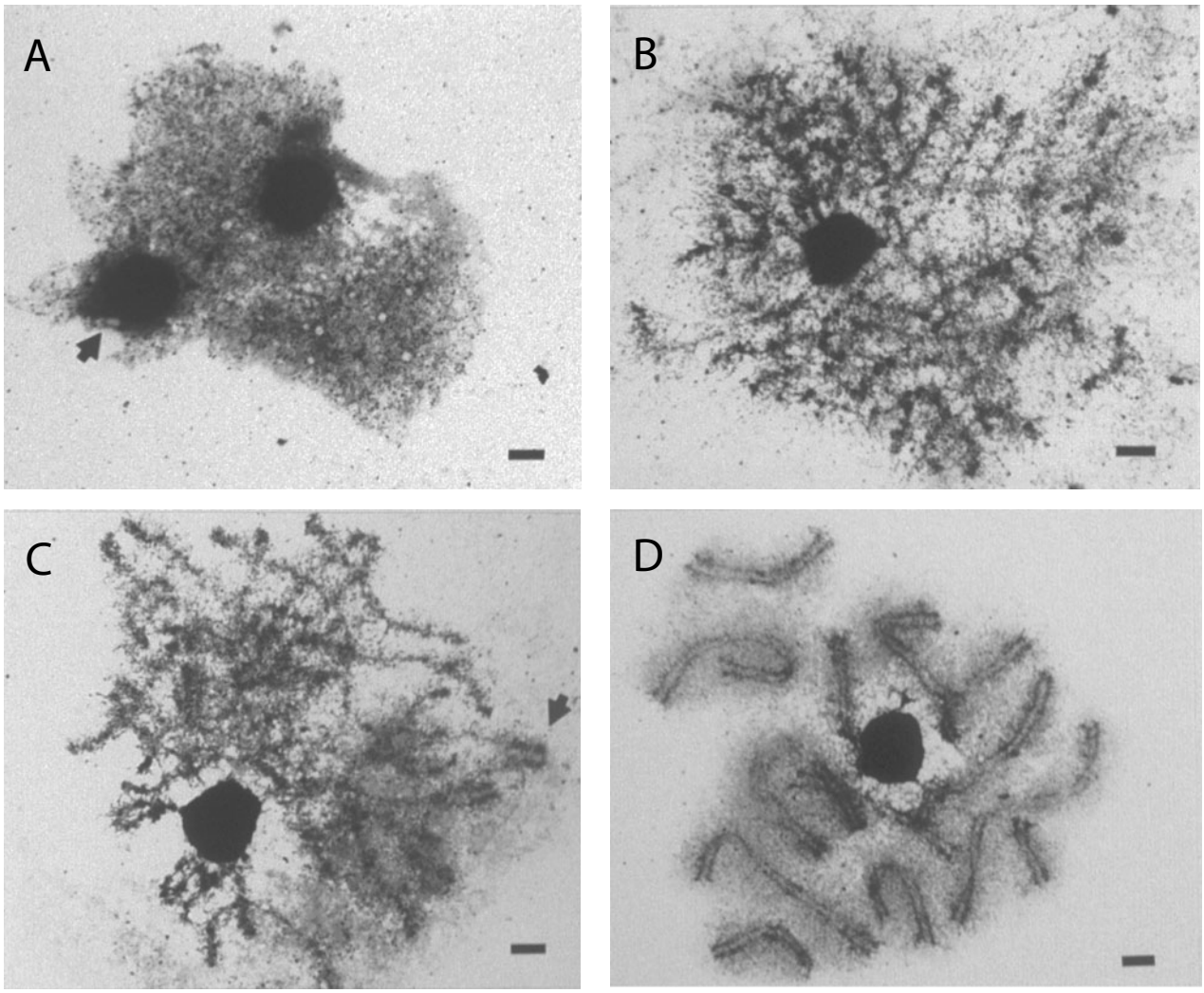

FIGURE 2 Electron microscopy of SC formation in *C. cinereus*. (A) At karyogamy, haploid nuclei fuse, and chromosomes are relatively uncondensed. Two nucleoli (dark structures indicated by an arrow) are apparent. (B) During leptotene, chromosomes condense and homologs start to pair. (C) During zygotene, chromosomes condense further and are paired along their length (note the pair in the upper right-hand corner of the image). The SC begins to form (arrow). (D) In pachytene, chromosomes are fully condensed, and homolog pairs are fully synapsed along their lengths. Scale bar, 1 μm. Reproduced from *Genetics* (Seitz et al., 1996), with the permission of the publisher.

and control of meiosis. For example, investigation of the *N. crassa* and *U. maydis* genomes shows interesting differences in orthologs of meiotic regulators (Borkovich et al., 2004; Donaldson and Saville, 2008). Meiotic regulation in *S. cerevisiae* and *S. pombe* is well characterized, with very little conservation of regulatory proteins (Honigberg and Purnapatre, 2003; Pawlowski et al., 2007). This highlights the drawbacks of concentrating research on a small number of organisms. Even within the relatively few other model systems widely used for the study of meiosis, such as *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*, differences in their meiotic processes are observed. For example, in most organisms (including *C. cinereus*) recombination is required for SC formation, but SC can form independently of recombination in *D. melanogaster* and *C. elegans* (discussed by Henderson and Keeney, 2005). Fungal research is well placed for exploitation of rapid-sequencing technologies; the relatively small size of fungal genomes (as an example, the *C. cinereus* genome is about 1/100 the size of the mouse genome) allows generation of very large amounts of data. These data, from many different fungi, will hopefully allow us to move towards an understanding of general, conserved meiotic processes.

CHIASMA DISTRIBUTION IN FUNGAL CHROMOSOMES

Chiasmata, which are the sites of reciprocal genetic exchange between two of the four chromatids in a bivalent, are not distributed randomly in most organisms. A random distribution of chiasmata would result in bivalents containing 0, 1, 2, etc., chiasmata with frequencies predicted by the Poisson distribution. Nonexchange bivalents would be prone to exhibit nondisjunction (the failure of chromosomes to separate properly) at the first meiotic division.

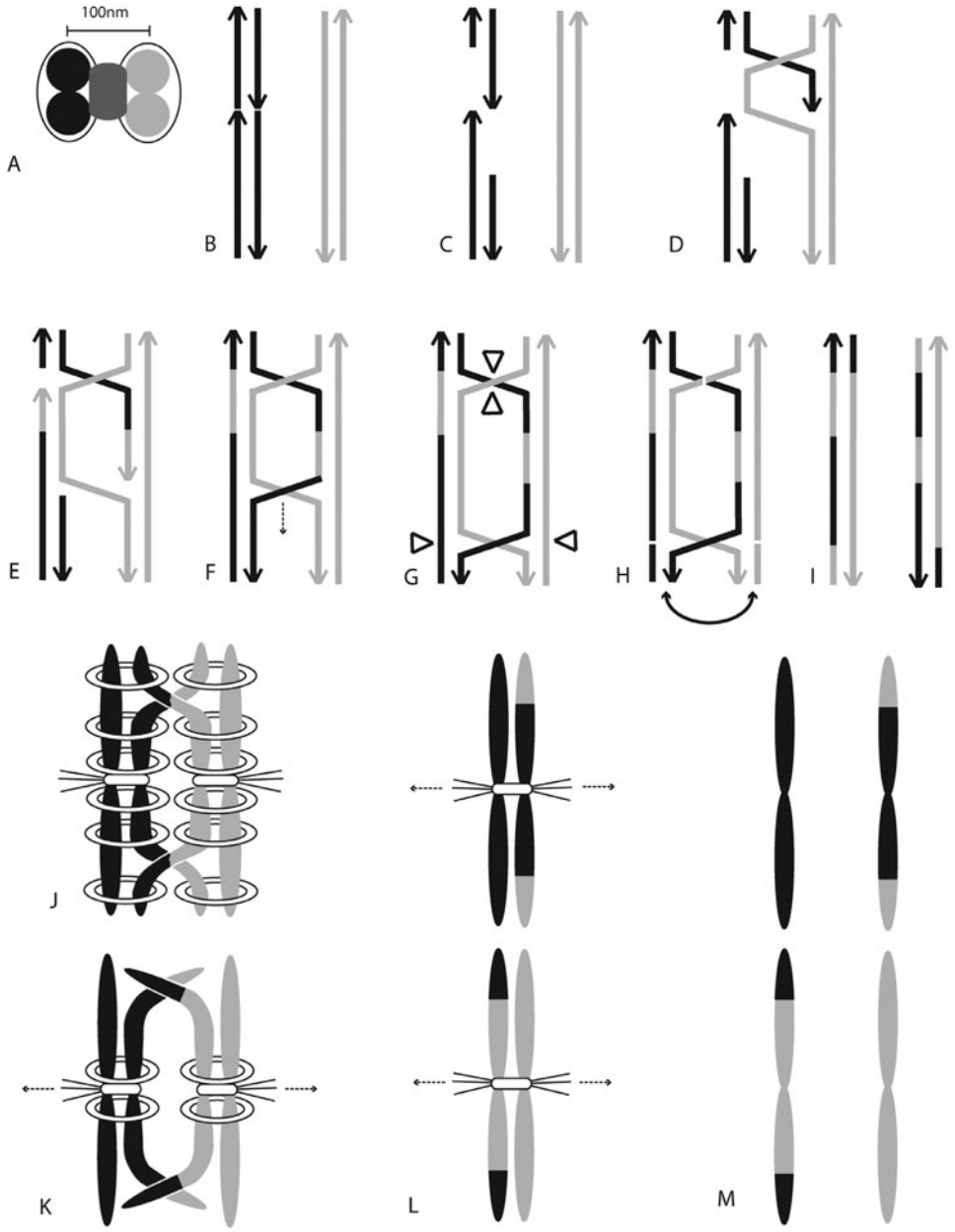

FIGURE 3 Formation of a crossover and its role in meiotic segregation. (A) The arrangement of sister chromatids and homologs, shown end-on. Sister chromatids (one pair in black and one pair in gray) are held together with cohesin (black ring). Homolog association is stabilized in many organisms by the SC (dark gray). This arrangement allows a sister from one homolog to interact with either sister from the second homolog. (B) Initiation of recombination. Spo11, a conserved protein, makes a DNA double-strand break, which is then resected in the 5′-to-3′ direction (C). To simplify, only one chromatid from each homolog is shown. Note that in panels B through I, both DNA strands are shown for the two chromatids. In panels J through M, all four chromatids are shown, each of which have two DNA strands (not shown), constituting eight strands in a pair of homologs. (D) The single strand invades a nonsister duplex, displacing a loop. The invading strand extends by replication, using the opposing duplex as template. The displaced loop may or may not be captured by the second single strand. If not captured, the invading strand and loop retract, and no crossover results. If captured, the second single strand expands, using the loop as a template (E). The invading strand is recaptured by the original sister, forming a double Holliday junction (F), which can then migrate (F and G). To resolve the junction, the DNA is nicked as shown by the arrows (G and H), forming a crossover (I). Note that the ends of the chromatids have exchanged, as represented by the different colors; this is a single crossover. (J) Crossovers are represented at the chromosomal level. Sister chromatids within homologs are shown, and chromosomes are compacted at this stage. Two crossovers are shown, as is typical in *C. cinereus*. Although the two crossovers are illustrated here as formed between the same chromatids, note that, due to the orientation illustrated in panel A, crossovers can and do form with either chromatid of the opposing homolog. Sister chromatids are held together by cohesin (rings), and this, combined with the crossover, is what holds homologs together while under tension at kinetochores. (K) Upon release of arm, but not centromere-associated, cohesin, homologs immediately begin to separate; this is the first meiotic division. For the second meiotic division, centromeric cohesin is released, allowing sister separation (L) and formation of the four meiotic products (M).

Sister chromatids would remain associated; sister centromeres would orient to one pole, but the bivalent would lack a connection between the homologs. The "balance of forces" necessary to orient the pairs of sister kinetochores to opposite poles would be impossible to establish without a connection between the homologs in the nonexchange bivalents. This situation could lead to meiotic arrest or to independent behavior of the two homologs. If they behave independently, one-half of the meioses will produce gametes aneuploid for the nonexchange chromosome.

Thus, it is not surprising that all organisms examined to date exhibit a nonrandom distribution of chiasmata. In particular, mechanisms exist in every organism that forms chiasmata to position these so that each bivalent contains at least one chiasma. However, beyond this generalization, there is a striking lack of uniformity in chiasma frequency per bivalent and in the location of chiasmata along the bivalents among all species in general and fungi in particular. Eukaryotes contain between 1 and 510 acrocentric, telocentric, metacentric, and/or holocentric chromosomes per genome (Boveri, 1887; Manton and Sledge, 1954). Fungi exhibit a narrower distribution of chromosome numbers and shapes in the genomes described to date, with a range of 3 to 20 mainly submetacentric chromosomes per genome (Gregory et al., 2007). Eukaryotes also exhibit a dramatic variation in DNA content (6.8 to 130,000 Mb/genome), while the range in fungi is again much narrower (6.8 to 795 Mb per genome [Gregory et al., 2007]). However, fungal genomes exhibit the **same** extremes in chiasma frequency per bivalent as are found in all eukaryotes (1 to 12 chiasmata per bivalent in fungi and 1 to 13 chiasmata per bivalent in all species [White, 1973]). These extremes are illustrated in Fig. 4, which compares two meiotic bivalents in prophase. The first (Fig. 4A) is from *S. cerevisiae* (Winzeler et al., 1998), and the second (Fig. 4B) is from *C. cinereus* (Stajich et al., unpublished data). The positions of all (or nearly all) the crossovers in *S. cerevisiae* and *C. cinereus* were determined by direct allelic variation scanning and simple sequence repeat mapping, respectively. Thus, an understanding of factors influencing the remarkably divergent chiasma distribution in experimentally tractable fungal model systems is likely to be highly relevant to other species of interest. Among the many ways that a specific chiasma distribution might have adaptive value, three appear to be the most relevant to understanding how specific chiasma distributions arise and are maintained. These three views of chiasma distribution are summarized here and considered in more detail in the following sections.

First, there appears to be a good correlation between sites of synaptic initiation and sites of chiasma formation. In fungi, there is a near-universal occurrence of subtelomeric sites of synaptic initiation. Several lines of evidence indicate that chiasmata also form at these subtelomeric sites. Taken together, these observations suggest that a key to understanding chiasma distribution in these species lies in understanding mechanisms that restrict synaptic initiation to particular chromosome regions (those that are clustered near the nuclear envelope during the "bouquet" stage).

Second, the placement of chiasmata relative to the centromere can have important physical consequences for chromosome shape. When chiasmata first become visible in diplotene, the bivalents are in an extended configuration, transcriptionally active, and flexible, and they can each lie flat in one plane. However, when chromosomes condense prior to the metaphase of the first meiotic division, the chiasmata define the boundaries of rigid loops that lie in planes perpendicular to each other, so the bivalents take on characteristic three-dimensional shapes. Since the kinetochores are attached to the spindle poles via microtubules, the chromatin between the kinetochores and proximal chiasmata on either side lies in the axial plane (parallel to the spindle microtubules). However, the distal chromatin (up to the next chiasma) forms a loop in the equatorial plane, followed by a loop in the axial plane, etc., in organisms with multiple chiasmata. Two extremes are illustrated at metaphase in Fig. 4. Very little chromatin lies in the axial plane in the *S. cerevisiae* bivalent with multiple proximal chiasmata (4A), while most of the chromatin lies in the axial plane in the *C. cinereus* bivalent with distal chiasmata (4B). Thus, the placement of the chiasmata has important consequences for orientation and shape of the bivalents on the meiotic spindle.

Third, chiasma localization influences the degree of linkage exhibited by adjacent markers. In some cases, the cosegregation of particular alleles in a haplotype segment

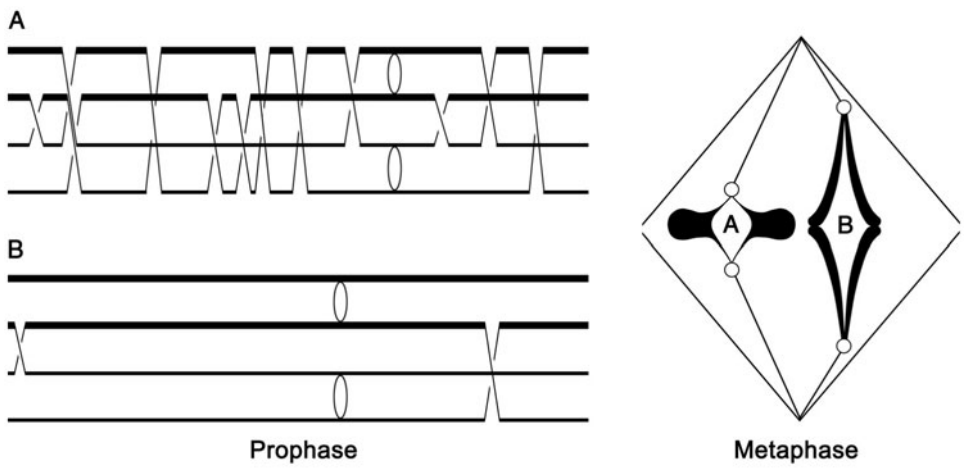

FIGURE 4 Variation in number of chiasmata per bivalent in fungi. (A) Meiotic bivalent from *S. cerevisiae* with 12 chiasmata diagrammed in prophase (left) and metaphase (right). (B) Meiotic bivalent from *C. cinereus* with two chiasmata diagrammed in prophase (left) and metaphase (right). Each chiasma results from a reciprocal exchange involving one chromatid from each homolog.

may be adaptive, while in others, the regular exchange in particular chromosome intervals may have selective value.

Subtelomeric Sites of Synaptic Initiation and Chiasma Formation in Fungi

Following the discovery by M. Moses of the SC in crayfish (Moses, 1956), fungi became favored material for cytological analysis because the relatively small size of fungal nuclei facilitated the serial sectioning and three-dimensional reconstruction of meiotic nuclei necessary to observe the assembly and structure of the SC (Gillies, 1972; Holm et al., 1981; Zickler, 1977). It was soon noted that synapsis initiated near chromosome ends, with the formation of patches of the tripartite, electron-dense SC near the nuclear envelope, where the chromosome ends were anchored. These patches then appeared to grow larger in a continuous fashion, with some interstitial sites of initiation noted also. Each initiation site was marked by a prominent electron-dense structure called a "recombination nodule" (RN) (Carpenter, 1975). Several lines of evidence indicate that synapsis initiates at sites where chiasmata will form. First, electron-dense material was observed to persist in regions initially marked by RNs, and the number and distribution of these regions corresponded to the number and distribution of chiasmata. Second, Maguire showed that the frequency of crossing-over within inversion heterozygosities in maize chromosomes was directly proportional to the frequency of homologous synapsis (via interstitial initiation) in the regions (Maguire, 1966; Maguire and Riess, 1994). Third, mutations that reduce the number of chiasmata also reduce the number of synaptic initiation sites (Zickler et al., 1992). Finally, mutations that abolish recombination in fungi also eliminate SC formation in most genetic backgrounds (Celerin et al., 2000; Keeney et al., 1997; reviewed by Zickler, 2006, and Zickler and Kleckner, 1999).

The occurrence of subtelomeric sites of synaptic initiation leads to an important prediction for the behavior of polyploid nuclei. As Loidl and Jones have pointed out (Loidl and Jones, 1986), switches in synaptic partners at the pachytene stage (when synapsis is complete) in these nuclei provide a record of the minimum number of independent sites that were used to initiate synapsis. In *C. cinereus*, analysis of triploid nuclei indicated that there are just over two sites per chromosome that can initiate synapsis (Pukkila et al., 1995). In *S. cerevisiae* tetraploid nuclei, despite a high overall recombination frequency (more than six chiasmata per bivalent), synaptic initiations were relatively infrequent. In fact, synapsis was initiated independently at more than one site in only one-half of the chromosome sets, producing an average of five quadrivalents per nucleus (Loidl, 1995). In both *S. cerevisiae* and in *C. cinereus*, the sites of independent synaptic initiation were usually subtelomeric, not interstitial. Recently, Kaback and collaborators (Barton et al., 2008) have used a new mapping technique and discovered that meiotic recombination rates are indeed elevated near the ends of *S. cerevisiae* chromosomes, as they are in *C. cinereus* (Stajich et al., unpublished). The similar levels of multivalent configurations in the two species despite the >3-fold difference in chiasma frequency raise the fascinating possibility of universal distinct properties of subtelomeric initiation sites in fungi in comparison to interstitial initiation sites. Further potential roles for the interstitial sites are considered below.

Chiasma Location and Chromosome Shape

Chiasmata can help to ensure proper chromosome disjunction by providing physical connections between homologous chromosomes. Since proper chromosome disjunction depends on the attachment of homologous centromeres to opposite poles at metaphase I, it is not surprising to learn that the location of these physical connections relative to the centromeres has important consequences for the efficiency of stable bipolar orientation of bivalents at metaphase I. In addition, the geometry of the spindle, the number of microtubules involved in pole-kinetochore attachments, the mechanisms of cohesin deposition around the centromeres (which must be maintained until metaphase II) and of deposition distal to the chiasmata (which must be released at metaphase I), the steps involved in signaling bipolar attachment of all bivalents (necessary to initiate anaphase I), and the physical properties of the surrounding nucleoplasm or cytoplasm are also variables that contribute to optimal chiasma localization.

The ways in which these variables interact to affect chiasma distribution is particularly obvious in plants and animals with large genomes and relatively small numbers of chromosomes (reviewed by White, 1973). For example, in the grasshopper *Stethophyma grossa*, with 13 acrocentric chromosomes, each bivalent in the male has a single proximal chiasma, so the bulk of the chromatin lies in the equatorial plane at metaphase I. In sharp contrast, the chiasmata in females are interstitial or distal, so the bulk of the chromatin lies in the axial plane with the narrow spindle surrounded by yolk masses (White, 1973). Sex-specific differences are also common in newts (Watson and Callan, 1963) and in plants such as *Lilium* and *Fritillaria* (Fogwill, 1958). The interplay of factors is clearly complex, since related species can show contrasting distributions in the two sexes. For example, in some newts, the males show localized chiasmata, while in others, the females display them (Watson and Callan, 1963). Interestingly, chiasma patterns in sex-reversed X-Y females are identical to those displayed in normal X-X females, suggesting the importance of the interplay of chromosome shape and spindle function (Wallace et al., 1997).

To date, analogous cytological studies have not been undertaken with fungi, since metaphase chromosome morphology using currently available techniques is poor. However, other lines of evidence point to the importance of chiasma location in proper disjunction of fungal chromosomes. Dawson and collaborators (Ross et al., 1996) used artificial "model chromosomes" and minichromosomes to examine the ability of a single chiasma at varying locations along the chromosome to promote proper disjunction. They observed that an exchange near a chromosome end was less likely to promote proper disjunction than an exchange closer to the centromere. It would appear that the many filamentous fungi that rely on one distal chiasma per arm must have mechanisms to insure that sister chromatid cohesion distal to the exchange is adequate to promote disjunction. In turn, this pattern of crossover distribution may facilitate bipolar orientation and subsequent anaphase onset, where very little "untangling" of chromatids would be required (Fig. 4B), in contrast to organisms that rely on multiple chiasmata (Fig. 4A).

Chiasma Location and Genetic Exchange

The occurrence of subtelomeric sites of crossing-over also makes an important prediction concerning relationships between genetic and physical maps. In many fungal species, the minimum map distance is 100 centimorgans (cM) $\times N$ (the haploid chromosome number), as predicted if there is usually a single reciprocal exchange (equivalent to 50 cM) per chromosome arm, plus varying levels of internal exchanges. In *C. cinereus*, the total map distance has been estimated to be 1,300 cM based on chiasma frequency (Holm

et al., 1981) and 1,346 cM based on a complete linkage map generated by using random amplified polymorphic DNA (RAPD) markers (Muraguchi et al., 2003). Since there are 13 chromosomes in *C. cinereus*, and since subtelomeric sites of synaptic initiation are the norm in this fungus (Holm et al., 1981), recombination hot spots and cold regions are predicted to occur in this species. The RAPD map is consistent with this prediction, since every chromosome showed a striking internal cluster of RAPD markers that were completely linked to each other. In fact, 91 of the 230 markers used fell into these clusters (3 to 18 markers per cluster). If the RAPD markers are randomly distributed in the genome, this result suggests that 40% of the *C. cinereus* genome experiences very little crossing-over. Recently, the prediction of subtelomeric hot spots and cold regions in the *C. cinereus* genome has been confirmed using a map based on simple sequence repeats. Stajich et al. (unpublished) found that nearly one-half of the crossovers occurred in subtelomeric hot spots. As predicted by the RAPD map, over one-half of the genome (53%) exhibited very little reciprocal exchange (3% of all exchanges).

In addition to the potential roles in synaptic initiation and chromosome shape described above, the potential adaptive value of genomes with large blocks exhibiting little genetic recombination together with other blocks exhibiting high rates of recombination deserves consideration. It has long been recognized that a reduced recombination rate would be advantageous when particular combinations of alleles at different loci provide a selective advantage (and/or other combinations provide a selective disadvantage) (Fisher, 1930; Kimura, 1956; Nei, 1967). Evidence that genes under such "epistatic selection" are in regions that are cold for recombination has also been obtained (Pal and Hurst, 2003).

The availability of a new set of genomic sequences together with anchored genetic maps from organisms with reduced recombination rates in comparison to the relatively small number of model systems analyzed to date should accelerate our understanding of how particular patterns of chiasma distribution along chromosomes arise and are maintained. Both the cold regions described above and hot spots are likely to be informative. For example, the mating type locus of *Cryptococcus neoformans* is flanked by two recombination hot spots (Hsueh et al., 2006). In addition to affecting the series of genome changes that enabled an ancestral tetrapolar mating system to evolve into the single complex MAT locus, the hotspots are undoubtedly instrumental in the spread of particular MAT loci to new genetic backgrounds. The molecular basis of the best-characterized fungal recombination hot spots is considered elsewhere in this volume (chapter 8).

GENES NECESSARY FOR MEIOSIS

How are conserved genes with roles in meiosis identified? Four methods have been employed. The first takes advantage of the growing number of fungal genome sequences now available, and genomic analyses, along with PCR-based approaches, have allowed the identification of a conserved core of meiotic genes (Table 1). Although a broad sampling of fungi is only currently in progress (J. Logsdon, personal communication), previous work shows that meiosis is certainly the ancestral state in fungi, as in all extant eukaryotes (Malik et al., 2008). Even though numerous fungi have no known sexual stage, their phylogenetic positions (Lobuglio et al., 1993) argue that if sex is not actually present it must have been lost in these lineages. In addition, population genetic data (for an example, see Burt et al., 1996; reviewed by Taylor et al., 1999) have shown that recombination is occurring even within putatively asexual lineages. Also, it was shown recently that Spo11 is required for homologous recombination in the *C. albicans* parasexual cycle (Forche et al., 2008); this is the only nonmeiotic role known for Spo11. Finally, in at least some cases the sexual stage may merely be cryptic to researchers; some species long thought to be asexual have recently been shown to have the hallmarks of sexual reproduction (Tzung et al., 2001; O'Gorman et al, 2009). Therefore, there is widespread conservation within fungi of core meiotic genes, and it will be interesting to determine the evolutionary and mechanistic relationships between meiotic sex and parasex, especially if they alternatively or coordinately adapt the same sets of proteins.

The second approach to the analysis of meiotic genes involves direct screens for meiotic mutants, either from wild-collected isolates or by laboratory mutagenesis. The analysis of wild strains of *N. crassa* (Leslie and Raju, 1985) showed that recessive mutations affecting meiosis are rampant in the wild. This is not unexpected in an organism that is not homothallic; if the meiotic genes are expressed only in the multinucleate or diploid phase, there will be no selection against mutations, since they will be complemented in the multinucleate or diploid phase.

There are numerous genes that will interfere with sporulation. For example, in *C. cinereus* a standard mutagenesis routinely yields about 1% survivors that form white mushrooms, indicating a defect in spore formation (Pukkila, 1994), but these are not always defective in meiosis (Kanda et al., 1989). Therefore, the absence of spore production does not necessarily indicate a defect in meiosis. Conversely, most meiotic mutants do cause defects in either spore formation or spore viability, although spore formation is usually more dramatically reduced in basidiomycetes (e.g., contrast *spo11* mutants in *S. macrospora* with those in *C. cinereus* [Celerin et al., 2000; Cummings et al., 1999; Storlazzi et al., 2003]). For *C. cinereus*, the failure of spore formation by meiotic mutants likely reflects both a regulatory coupling between successful completion of meiosis and spore development and also gene expression contributions from the haploid meiotic products to the developing spores.

The third method of identification of genes necessary for meiosis is to take advantage of the evolutionary and biochemical connections between meiosis and mitotic processes, most notably DNA double-strand break repair (reviewed by Cummings and Zolan, 1998; Goldman et al., 2002; and Pukkila, 1994). For example, by use of this approach, mutations in the genes encoding proteins of the conserved MRN complex (Mre11, Rad50, and Nbs1) have been found to disrupt meiosis in both filamentous fungi (Acharya et al., 2008; Gerecke and Zolan, 2000) and yeasts (Alani et al., 1990; Johzuka and Ogawa, 1995; Young et al., 2004; reviewed by Borde, 2007). The Rad51 protein, a eukaryotic homolog of the bacterial RecA protein, was shown to be required for meiosis in *A. nidulans* (van Heemst et al., 1997), and it associates with meiotic chromosomes in filamentous fungi with timing consistent with its role in meiotic recombination (Acharya et al., 2008; Tesse et al., 2003) (Fig. 5).

The fourth method for the identification of meiotic genes takes advantage of modern genomics to examine global patterns of gene expression. This approach and its contributions to date are summarized in the last section of this chapter.

TABLE 1 Some core meiotic genes and their functions and conservation among fungi[a]

Gene	Presence of orthologs in completed sequencing project for:												Protein function
	Sc	*Cg*	*Kl*	*Ca*	*Sp*	*Nc*	*Gz*	*Mg*	*An/f/o*	*Um*	*Cn*	*Cc*	
spo11	+	+	+	+	+	+	+	+	+	PA	+	+	Transesterase, creates DNA DSBs in meiotic chromosomes
Mre11	+	+	+	+	+	+	+	+	+	+	+	+	3′–5′ double-stranded DNA exonuclease and single-stranded DNA endonuclease, forms complex with Rad50 and Nbs1, required for DSB processing
rad50	+	+	+	+	+	+	+	PA	+	+	+	+	SMC protein, ATPase, forms complex with Mre11 and Nbs1
hop1	+	+	+	+	+	−	−	PA	+	PA	+	+	DNA binding protein that displays Red1-dependent localization to the unsynapsed axial-lateral elements of the synaptonemal complex; required for homologous chromosome synapsis and chiasma formation
hop2	+	+	+	+	+	−	−	PA	+	PA	+	+	Protein that localizes to chromosomes, preventing synapsis between nonhomologous chromosomes and ensuring synapsis between homologs; complexes with Mnd1p to promote homolog pairing and meiotic DSB repair
mnd1	+	+	+	+	+	−	−	PA	+	PA	+	+	With Hop2, functions after meiotic DSB formation, and required for stable heteroduplex DNA formation
dmc1	+	+	+	+	+	−	−	PA	+	PA	+	+	Meiosis-specific homolog of Rad51, has similar function but promotes interhomolog recombination
rad51	+	+	+	+	+	+	+	+	+	+	+	+	Forms helical filaments on single-stranded and double-stranded DNA and catalyzes homologous DNA synapsis and strand exchange
msh4	+	+	+	+	−	−	+	+	+	+	+	+	Forms a heterodimer with Msh5, interacts with Mlh1/Mlh3 heterodimer. Required for crossover recombination
msh5	+	+	+	+	−	+	+	+	+	+	+	+	Forms a heterodimer with Msh4, interacts with Mlh1/Mlh3 heterodimer. Required for crossover recombination
mer3	+	+	+	+	−	+	−	+	+	+	+	+	Meiosis-specific DNA helicase
rad21 (scc1)	+	+	+	+	+	+	+	+	+	+	+	+	Holds Smc1 and Smc3 heads together by binding N-terminal domain to Smc3 and C-terminal domain to Smc1, forming cohesin ring
rec8	+	+	+	+	+	+	+	+	+	+	+	+	Meiosis-specific paralog of Rad21

Meiosis-specific genes are in bold. Symbols and abbreviations: +, orthologs are present; −, orthologs are absent; PA, orthologs are putatively absent; *Sc*, *Saccharomyces cerevisiae*; *Cg*, *Candida glabrata*; *Kl*, *Kluyveromyces lactis*; *Ca*, *Candida albicans*; *Sp*, *Schizosaccharomyces pombe*; *Nc*, *Neurospora crassa*; *Gz*, *Gibberella zeae*; *Mg*, *Magnaporthe grisea*; *An/f/o*, *Aspergillus nidulans/fumigatus/oryzae*; *Um*, *Ustilago maydis*; *Cn*, *Cryptococcus neoformans*; *Cc*, *Coprinus cinereus*. Data are from Malik et al., 2008, and Malik and Logsdon (unpublished). See Malik et al. (2008) and references therein for a comprehensive survey of meiotic genes.

Comparative Studies of Meiosis in Filamentous Fungi

Studies of mutants in filamentous fungi have made substantial contributions to the analysis of the two core features of meiosis: the structure of meiotic chromosomes and the role of interhomolog crossovers in meiotic chromosome structure and behavior.

Meiotic Chromosome Structure Is Set up during Meiotic S Phase

The DNA replication that precedes meiosis (premeiotic S) is unique in that it is the critical time during which chromosomes are prepared structurally for interhomolog recombination (Forsburg, 2002). For some fungi, including *C. cinereus* and *N. crassa*, it has been documented that premeiotic S

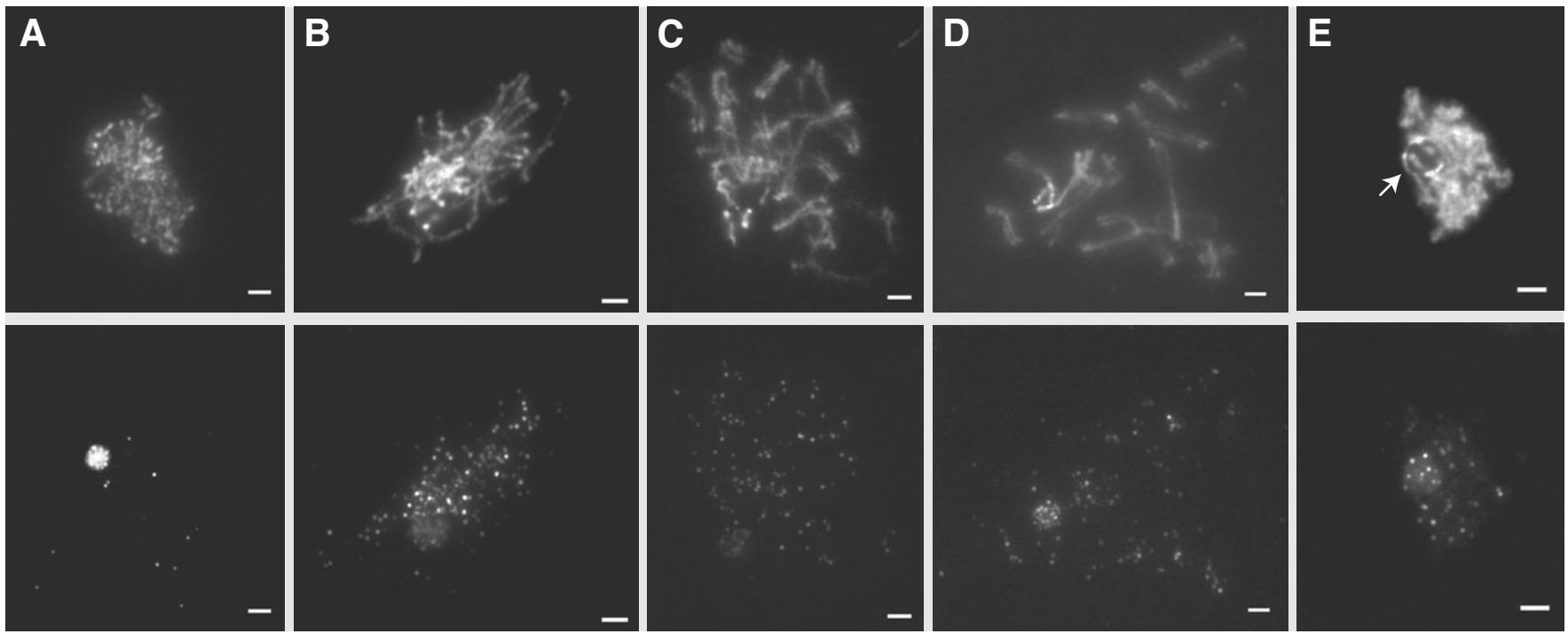

FIGURE 5 Time course of Rad51 association with meiotic chromosomes. Chromosome spreads were stained with DAPI (4′,6-diamino-2-phenylindole) (top panels) and antibody against *C. cinereus* Rad51 (bottom panel), which was detected using fluorescein isothiocyanate-labeled antirabbit antibody. (A) Preleptotene image taken 1 h after karyogamy (K+1). (B) Leptotene image taken at K+2. (C) Zygotene image taken at K+4. (D) Pachytene image taken at K+6. (E) Diplotene image taken at K+8. Arrows in panel E show ribosomal DNA, which does not synapse, within the nucleolus. Scale bars represent 2 μm for all images. All panels except panel A are reproduced from *Genetics* (Acharya et al., 2008), with the permission of the publisher.

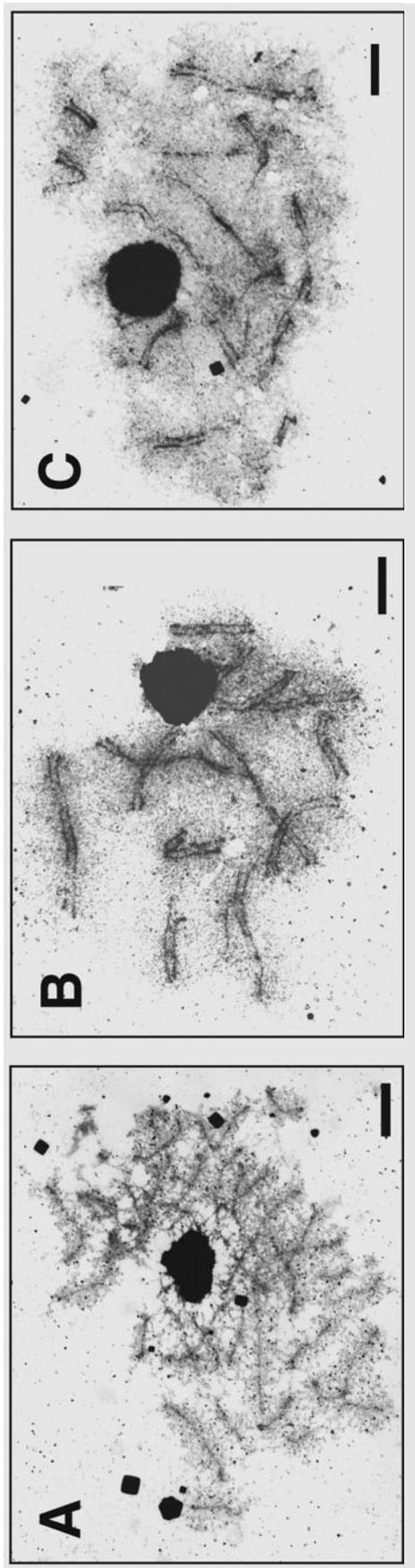

FIGURE 6 The *spo22-1/msh5-22* mutation suppressed synapsis defects of *spo11-1*. Surface spreads of meiotic chromosomes were stained with silver nitrate and photographed using transmission electron microscopy. (A) *spo11-1*; (B) *spo22-1/msh5-22*; (C) *spo11-1 msh5-22* double mutant. Scale bars represent 2 μm for all images. All images are reproduced from Merino et al. (2000), with the permission of the publisher.

occurs prior to karyogamy (Iyengar et al., 1977; Kanda et al., 1990). Therefore, events that are critical parts of prophase I actually occur before the diploid nucleus forms. In other fungi, the timing of premeiotic S is less clear; evidence now exists that in *Magnaporthe grisea* it occurs postkaryogamy (Farman, 2002).

The fundamental difference between a mitotic G_2 chromosome and a chromosome entering meiosis is seen in the relationship between the two sister chromatids (reviewed by Petes and Pukkila, 1995, and Zickler, 2006). Three features are particularly noteworthy. First, meiotic crossing-over is suppressed between sister chromatids except within the rRNA genes, which do not synapse (Fig. 5E) (Petes and Pukkila, 1995). Second, an increased need for sister chromatid cohesion is imposed by recombination. This is seen by recruitment of extra cohesin to double-strand break sites during mitosis (Strom et al., 2004; Unal et al., 2004) and by the suppression of cohesion defects by mutations that prevent recombination (Storlazzi et al., 2003, 2008). Third, cohesion must be maintained at the centromere during anaphase I, to allow sister chromatids to stay together through the first meiotic division (Fig. 3). The last step, the protection of cohesin at the centromere, is effected by the substitution of the mitotic cohesin component Rad21/Scc1 with a meiosis-specific paralog, Rec8 (Watanabe and Nurse, 1999). The association of Rec8 with other proteins allows it to escape cleavage at anaphase I (Fig. 3) (Kitajima et al., 2004; Watanabe and Kitajima, 2005).

A mutant of *C. cinereus*, initially called *spo22-1* (Kanda et al., 1990), has allowed an unusual insight into both the role of sister chromatids in meiosis and the requirement that sister chromatids impose for the activities of other meiotic genes. In *spo22-1*, premeiotic DNA replication is specifically inhibited (Kanda et al., 1990). Although it is not yet known whether replication initiates and then falters, cytogenetic data make it clear that there are functionally no sister chromatids in the *spo22-1* mutant (Pukkila et al., 1995). The *spo22-1* mutant has recently been shown to be an allele of *msh5*, one of the six eukaryotic *mutS* homologs in *C. cinereus* (Celerin et al., unpublished data). The gene *msh5* was initially identified as specifically required for crossover formation (Hollingsworth, 2008), and most models for its function place it in Holliday junction resolution (Snowden et al., 2004, 2008), in partnership with Msh4, with which it forms a dimer. However, Msh5 is also required early in meiosis for the specification of recombination sites as crossover sites (Borner et al., 2004); see Bishop and Zickler, 2004, for an excellent review. So far, its role in premeiotic DNA replication is known uniquely in *C. cinereus*. However, it is possible that other filamentous fungi, in which premeiotic S precedes karyogamy, will show a similar Msh5 dependency.

Role of Recombination Proteins

Meiotic recombination initiates with a DNA double-strand break made by the protein Spo11, which is homologous to archaebacterial topoisomerases (Keeney, 2007; Keeney et al., 1997) (Fig. 3). In the filamentous fungi examined, as well as in *S. cerevisiae* and mouse, the chromosomes of *spo11* mutants condense and can form complete axial cores (Fig. 6) (Baudat and de Massy, 2007; Bellani et al., 2005; Celerin et al., 2000; Keeney et al., 1997; Storlazzi et al., 2003) but essentially never form SC. Therefore, the dependency of synapsis on recombination articulated for *S. cerevisiae* (Alani et al., 1990; Padmore et al., 1991) seems to be true for the filamentous fungi. Spo11 requires the functions of other proteins for its break-forming activity, although whether for direct participation in the reaction or

because of prebreak effects on chromatin is not always clear (reviewed by Keeney, 2007). In *S. cerevisiae*, the protein Ski8, initially identified as required for degradation of nonadenylated mRNAs, is required for Spo11 function and interacts with it in yeast two-hybrid assays (Arora et al., 2004; Uetz et al., 2000). For *S. macrospora*, Tesse et al. (2003) showed that the Ski8 and Spo11 proteins are interdependent for localization to meiotic chromosomes and that *ski8* mutant phenotypes are similar to those in strains defective for *spo11* and can be similarly suppressed by irradiation-induced DSBs. An interesting aspect of this study was the analysis of an allelic series of loss-of-function mutants of *ski8*. These mutants were examined for their ability to form Rad51 foci, an indicator of recombinational activity. A direct correlation was seen between nuclei that had more Rad51 foci and those that progressed further in synapsis.

A result similar to that found for *ski8* mutants was seen in an allelic series of *rad50* mutants of *C. cinereus* (Acharya et al., 2008). In contrast to the situation for *S. cerevisiae*, DSB formation is not Rad50 dependent in *C. cinereus*, but DSB processing requires Rad50. The *rad50* alleles that form more Rad51 foci undergo significantly more synapsis and also increased stable homolog pairing. Therefore, the mechanistic link between recombination progression and homolog synapsis is conserved in filamentous fungi.

In *C. cinereus*, *msh5* mutations have been employed as tools for the examination of the links between recombination and synapsis. For example, both *spo11* and *rad50* mutants show significantly more synapsis when an *msh5* mutation is introduced (Fig. 6) (Merino et al., 2000; F. M. Kennedy, O. P. Savytskyy, and M. E. Zolan, unpublished data). Remarkably, when homolog pairing is assayed using fluorescence in situ hybridization, the *spo22-1/msh5-22* mutation completely suppresses the need for Rad50 (Kennedy et al., unpublished). These studies lead to two conclusions. First, the requirement for Spo11 activity in synapsis is replication dependent. Second, there are very few Rad51 foci formed in *msh5-22*; the level is not significantly different from that of the *spo11-1* mutant. Therefore, either the small number of Spo11-independent Rad51 foci indicates recombinational sites that are insufficient to promote synapsis in the presence of a sister (i.e., in the *spo11-1* single mutant) but are sufficient for synapsis in the absence of a sister chromatid, or the wild-type Msh5 protein prevents synapsis in the absence of recombination, and this block is removed in *msh5* mutant strains.

Transcriptional Regulation

The availability of ever-increasing numbers of fungal genomes, with concomitant tools such as microarrays, has allowed genome-wide analyses of transcription under many conditions (for a summary of early fungal array studies, see Breakspear and Momany, 2007). The areas of study discussed in that review include metabolism, symbiosis, pathogenesis, and development, which includes fruiting body production and conidiation but no scrutiny of meiosis. Transcriptional regulation of meiosis has been profiled in *S. cerevisiae* (Chu et al., 1998; Primig et al., 2000) and *S. pombe* (Mata et al., 2002), but data from filamentous fungi are scarce. Genes expressed in fungal fruiting bodies have been profiled by array analysis in few fungi, including *S. macrospora* (Nowrousian et al., 2005) and *Fusarium graminearum* (Qi et al., 2006), but to date specific investigation of meiotic transcription is limited to *U. maydis* (Zahiri et al., 2005) and *C. cinereus* (Burns et al., unpublished data).

Data from *U. maydis* identified only a small number of meiotically regulated transcripts. This is in contrast with the transcriptional studies in *S. cerevisiae* (Chu et al., 1998; Primig et al., 2000), *S. pombe* (Mata et al., 2002), and *C. cinereus*, which indicate that a large proportion of the genome is up- or down-regulated throughout meiosis and sporulation; this is unsurprising given the substantial biochemical and physiological changes required during a short time frame. Clustering of genes with similar expression profiles is indicative of coordinate function and regulation (Eisen et al., 1998). Gene clustering of meiotic and sporulation time course experiments identified successive waves of transcription, with seven or eight clusters (*S. cerevisiae*) or four clusters (*S. pombe*) describing stress and response to nutritional changes (induction of meiosis), premeiotic S and prophase I, meiotic divisions, spore formation, and spore maturation. Transcription data from gill tissue of *C. cinereus*, facilitated by the synchronous development of meiotic tissues in this organism, produce nine clusters that also exhibit transcriptional waves. This 15-h time course experiment encompasses data from prekaryogamy, meiotic S phase, meiotic prophase, meiotic divisions, and the formation of tetrads, but not sporulation.

The transcriptional profiles of meiotic progression in *C. cinereus* bear considerable similarities to those from *S. cerevisiae* and *S. pombe*. The most striking transcriptional change in all three fungi occurs after expression of prophase-I-specific genes, such as those involved in creating double-strand breaks, maintaining sister chromatid cohesion, and formation and resolution of crossovers. Subsequently, there is a wholesale downregulation of these meiotic genes and a concomitant switch to upregulation of genes associated with sporulation, such as those involved in production of energy reserves, spore budding, and metabolism of cell wall components such as hydrophobins.

Expression profiling studies, in concert with gene ontology analysis and comparison with orthologs from other organisms, are extremely important for discernment of timing and function of meiotic genes. Such approaches have been used successfully with *S. pombe* and *S. cerevisiae* to identify new genes involved in meiosis and sporulation (Enyenihi and Saunders, 2003; Gregan et al., 2005; Jordan et al., 2007; Martin-Castellanos et al., 2005; Rabitsch et al., 2001). Further scrutiny of meiotically expressed genes will aid discovery of poorly conserved genes, such as those for the SC. In addition, transcriptional profiling of meiotic mutants will further elucidate pathways and functions.

Regulation of Meiosis

One area ripe for further investigation is transcriptional control of meiosis in filamentous fungi. Extensive studies involving *S. cerevisiae* and *S. pombe* have revealed little similarity in the mechanisms of meiotic regulation, yet in both species meiosis is induced by nutritional restriction. In *S. cerevisiae*, the master regulator of early meiotic genes is IME1, via UME6, which binds to an upstream motif common to many of these genes. Subsequent gene expression is controlled by NDT80, and these genes again bear common upstream motifs (Chu et al., 1998; Primig et al., 2000). In contrast, no common motifs have yet been discovered in the upstream regions of coordinately expressed meiotic genes in *C. cinereus*. The IME1 equivalent in *S. pombe*, STE11, bears no resemblance to the *S. cerevisiae* protein yet has a similar role in responding to nutritional deficiencies (Mata et al., 2002). Neither of these proteins has orthologs in *C. cinereus*; the light-induced meiosis in *C. cinereus* deems this very unlikely.

Similarly, other fungi in which meiosis and sporulation are differently induced may also be likely to employ different regulatory mechanisms. Although these main regulatory

proteins have no C. *cinereus* orthologs, some other aspects of the regulatory machinery, such as cyclin-dependent kinases, are present. Intriguingly, Mre11, part of the meiotic complex that processes double-strand breaks, has been implicated in transcriptional regulation of further meiotic and sporulation genes in *S. cerevisiae* (Kugou et al., 2007); *mre11* expression in C. *cinereus* is unexpectedly slightly earlier than that of another complex member (*rad50*), and it is possible that further investigation could reveal a regulatory role. A number of putative transcription factors were identified in transcriptional profiling of C. *cinereus*; these may perform functions similar to those of the regulators from yeast species.

In addition, several filamentous fungi have orthologs of *S. pombe* Mei2, which has been shown to sequester meiotic transcripts in vegetative growth (Harigaya et al., 2006). Meiotic transcripts have also been shown to undergo regulated splicing in yeast meiosis (Juneau et al., 2007), indicating a further level of regulation; genes in *S. cerevisiae* have few introns, with meiotic genes containing a disproportionately high number. Meiotic genes in C. *cinereus* appear to contain a higher number of introns than other genes, and a number of RNA-splicing factors are expressed throughout prophase I, perhaps suggesting a level of intron-mediated regulation; this could be probed using high-density microarrays or next-generation sequencing approaches. Regulation of meiosis at the epigenetic level, manipulation of recombination hot spots, and targeted disruption of cohesion complex components are also exciting targets for future analyses of the meiotic process in filamentous fungi.

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8

DNA Repair and Recombination

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To maintain the integrity of the genome, all organisms must continually monitor their chromosomes and repair any lesions. DNA damage can occur in many ways: through exposure to radiation, including UV and ionizing radiation (IR); as a result of the activity of chemical mutagens, such as methyl methanesulfonate (MMS); and during internal processes such as replication and recombination. Yet diversification of gene sequences by mutation and the subsequent shuffling of novel sequences into new combinations is required for evolution to occur, allowing species to adapt to environmental change and to develop new complexity and capabilities.

Recombination yielding genetic diversity occurs during the pachytene stage of meiosis (see chapter 7 in this volume), the reductional division that leads to the formation of sperm and eggs in mammals or the haploid spores of filamentous fungi. However, recombination can also occur in vegetative cells, where it is part of the armory of repair systems available to correct mutational damage.

Investigations using filamentous fungi have made major contributions to our understanding of the core biological processes of DNA repair and recombination, as certain model species provide particularly favorable opportunities for insight. The Ascomycete fungi, including the filamentous fungus *Neurospora crassa*, conveniently provide a full set of the products of a single meiosis packaged in spores inside a single ascus. Both *N. crassa* and *Sordaria fimicola* also have the advantage of spores that are ordered within the ascus, allowing convenient detection of marker segregation at the first or second division of meiosis, or in the subsequent mitotic division, which reflects a failure of repair of mismatches in heteroduplex DNA.

Analysis of *Neurospora* spores provided the first evidence that recombination is not explained just by breakage of homologous chromosomes at equivalent places and reciprocal exchange of the broken ends during repair of the breaks. Mary Mitchell (1955) found that instead of a 4:4 (Mendelian) segregation of allelic genes in a heterozygous cross, there was often a 6:2 segregation, as if one allele had been converted into the other. Further work with *Neurospora* nutritional mutants (Case and Giles, 1958a; Stadler, 1958; Murray, 1960) and with spore color markers in *Sordaria* (Olive, 1959; Kitani et al., 1962) and in *Ascobolus immersus* (Lissouba et al., 1962) contributed to the development of the first plausible molecular explanation for the events of meiotic recombination (Holliday, 1964). However, the demonstration that meiotic gene conversion occurs in *Saccharomyces cerevisiae* (Fogel and Hurst, 1967), a yeast species of economic importance commanding the resources for early determination of its genome sequence, has resulted in work with yeast leading the way to uncovering details of basic processes of recombination.

DNA REPAIR

The study of DNA repair began with the discovery of photoreactivation (Kelner, 1949) and UV-sensitive mutants in bacteria (Hill, 1958), followed by isolation and characterization of mutants with sensitivity to UV, IR, and chemical mutagens in both *Escherichia coli* and the yeast *S. cerevisiae*. Mutagen-sensitive yeast mutants were characterized by analysis of the degree of sensitivity to particular mutagens and of epistasis in double mutants (Game, 2000). Three major groups of mutants were identified, each with a different role in DNA repair: *excision repair*, which removes damaged DNA such as thymine dimers; *recombination repair*, which repairs DNA strand breaks; and *postreplication repair*, which fills DNA gaps after replication.

DNA repair in *Neurospora* (Schroeder et al., 1998) and *Aspergillus nidulans* (Kafer and May, 1998) appears more complex than in yeast. Early attempts to group six UV-sensitive *N. crassa* mutants (*upr-1*, *uvs-2*, *uvs-3*, *uvs-4*, *uvs-5*,

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and *uvs-6*) as had been done in *S. cerevisiae* were unsuccessful. For example, the *N. crassa uvs-2* gene was initially thought to be involved in nucleotide excision repair (NER), because the mutant phenotype is similar to that of *S. cerevisiae* NER mutants. However, when *uvs-2* was cloned and sequenced, it was found to encode a homolog of *S. cerevisiae* Rad18, with both ring finger and zinc finger motifs (Tomita et al., 1993), indicating a role in postreplication repair (PRR) rather than NER. The *N. crassa mei-3* gene, whose mutants were originally characterized as mutagen sensitive and defective in meiosis (Newmeyer and Galeazzi, 1977, 1981), was found to be a homolog of *E. coli recA* and *S. cerevisiae RAD51* (Cheng et al., 1993; Hatakeyama et al., 1995). Since *uvs-6* is epistatic to *mei-3*, the gene products are in the same pathway of recombination repair. In addition, it has recently been shown that *uvs-3* is involved in damage checkpoint control (Kazama et al., 2008).

Since NER mutants with high sensitivity to UV were not found in fungi, early DNA repair mutants were mainly isolated as strains showing high sensitivity to MMS or other chemical mutagens. More recently, utilization of the *N. crassa* genome database (Galagan et al., 2003) has made it possible to identify additional DNA repair genes (Table 1) (Borkovich et al., 2004), but many of the phenotypes have yet to be characterized by experimentation.

NER Pathways in Filamentous Fungi

Discovery of a *mus-18* mutant sensitive only to UV suggested that *N. crassa* has two different NER systems (Hatakeyama et al., 1998; Ishii et al., 1998). The *N. crassa mus-38*, *mus-43*, and *mus-44* genes, homologs of *S. cerevisiae RAD1*, *RAD14*, and *RAD10*, respectively, are components of the NER pathway common to most other organisms (Hatakeyama et al., 1998; Sato et al., 2008), but *mus-18* forms part of a novel NER pathway (Ishii et al., 1991), and the redundancy in NER gives *N. crassa* high resistance to UV. *mus-18*, which encodes a UV damage-specific endonuclease (Yajima et al., 1995), although lacking in most higher organisms including *S. cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, has homologs in many fungi including *Aspergillus*, *Coprinus*, and *Ustilago*.

PRR Pathways

Mutants belonging to the PRR pathway group are extremely sensitive to UV, mitomycin C, and MMS, but their sensitivity to hydroxyurea (HU), an inhibitor of ribonucleotide reductase, is similar to that of the wild type. *N. crassa uvs-2* and *mus-8*, induced by DNA damage, are homologs of *S. cerevisiae RAD18* and *RAD6* and encode a ubiquitin ligase (E3) and ubiquitin-conjugating enzyme (E2), respectively (Tomita et al., 1993; Soshi et al., 1996). In *S. cerevisiae*, these enzymes are recruited to a DNA lesion at which DNA polymerase is stalled and monoubiquitylate Lys164 of proliferating cell nuclear antigen, the DNA sliding clamp (Hoege et al., 2002). This is a key step of PRR and enhances the survival of cells with persistent unrepaired DNA damage by regulating the mechanisms of template switching and translesion DNA synthesis (TLS). *S. cerevisiae* Srs2, a 3′-to-5′ DNA helicase, dissociates Rad51-nucleoprotein filaments and prevents repair by the homologous recombination (HR) pathway, resulting in repair by TLS (Krejci et al., 2003, Veaute et al., 2003). In contrast, the *N. crassa* Srs2 homolog, Mus-50, has a different function, unraveling intertwined DNA during the recombination process (Suzuki et al., 2005). In *S. cerevisiae*, the Ubc13/Mms2/Rad5 complex extends the ubiquitin chain from the monoubiquitylated proliferating cell nuclear antigen with a noncanonical lysine 63-linked ubiquitin-chain (Hoege et al., 2002). *S. cerevisiae* Rad5 is a ubiquitin ligase (E3), essential for the polyubiquitylation, but the *N. crassa RAD5* homolog, *mus-41*, is not required for polyubiquitylation (Kawabata et al., 2007). The *mus-41* mutant is sensitive to UV and MMS, and *uvs-2* is epistatic to *mus-41*. *N. crassa* has Ubc13 and Mms2 homologs, Mus-46 and Mus-47, respectively. The single mutants, *mus-46* and *mus-47*, and the *mus-46 mus-47* double mutant are each equally sensitive to UV and MMS, and *uvs-2* is epistatic to both, suggesting a direct interaction of Mus-46 and Mus-47, which has been confirmed by a yeast two-hybrid assay (Kawabata et al., 2005).

Both filamentous fungi and mammals have a greater number of TLS polymerases than does *S. cerevisiae*. *N. crassa upr-1* and *mus-26*, mutants, which are UV sensitive but show low mutability (Sakai et al., 2002, 2003), are in the *uvs-2* subgroup. *upr-1* and *mus-26* are homologs of *S. cerevisiae Rev3* and *Rev7* and encode the catalytic subunit and associated protein of TLS polymerase ζ, respectively. Polymerase η, encoded by the human XP variant gene, is also a TLS polymerase. *Neurospora* and *Aspergillus* have several additional genes that appear to be TLS polymerase homologs (Borkovich et al., 2004), but the roles of these are not yet known.

Recombination Repair

Genes involved in recombination repair form two groups, differentiated by the phenotype. DNA double-strand breaks (DSB) are repaired by two major pathways: HR and nonhomologous end joining (NHEJ). These pathways are regulated by the same upstream process (Fig. 1).

HR Repair

N. crassa mei-3, *mus-11*, and *mus-25* mutants are sensitive to MMS, bleomycin, histidine, and IR and, except for *mus-11*, are also slightly sensitive to UV. Homologs are widely distributed from yeast to higher organisms and correspond to *S. cerevisiae RAD51*, *RAD52*, and *RAD54*, respectively (Borkovich et al., 2004). Despite the sequence similarity, phenotypes of the mutants vary between species for each homolog. For example, the *S. cerevisiae rad52* mutant is highly sensitive to many mutagens and sterile in homozygous crosses (Game, 1993; Shinohara and Ogawa, 1995), similar to the *N. crassa mus-11* mutant, but the vertebrate *RAD52* mutant is insensitive to mutagens and fertile (Rijkers et al., 1998), suggesting that Rad52 may have a different function in each organism. In *S. cerevisiae*, Rad52 removes the single-strand binding protein RPA to allow access to Rad51 (Haber, 1999). In addition, orthologs of the human *BRCA1* and *BRCA2* genes, which play important roles in homologous recombination, have not been found in lower eukaryotes, including *S. cerevisiae*, *N. crassa*, and *A. nidulans*.

As indicated above, sequence similarity between *N. crassa mei-3*, *mus-11*, and *mus-25* and their *S. cerevisiae* orthologs does not always signify functional similarity. These mutants are sterile in homozygous crosses, preventing assessment of their effect on meiotic recombination, and since the *Neurospora* vegetative cell is haploid and includes little repetitive DNA, mitotic recombination is a rare event. However, if DNA fragments with sequence homologous to part of the genome are transformed into the wild type, 3 to 10% of the transformants result from homologous integration. In contrast, if *mei-3*, *mus-11*, or *mus-25* mutants

TABLE 1 DNA repair pathways

Repair pathway	*S. cerevisiae* gene	Function	*N. crassa* homolog	Homolog in other fungi	Reference
NER	*RAD1*	5′ endonuclease	*mus-38*	AN8713.3[a]	
	RAD2	3′ endonuclease	*mus-40*	AN5216.3[a]	
	RAD10	5′ endonuclease	*mus-44*	AN4331.3[a]	
	RAD14	Damaged DNA binding activity	*mus-43*	AN3784.3[a]	
	uve1^{+}[b]	UV damage-dependent endonuclease	*mus-18*	AN0604.3[a]	
PRR	*RAD5*	Ubiquitin ligase (E3)	*mus-41*	AN0044.3[a]	
	RAD6	Ubiquitin conjugating enzyme (E2)	*mus-8*	*A. nidulans uvsJ*	Goldman and Kafer, 2004
	RAD18	Ubiquitin ligase (E3)	*uvs-2*	*A. nidulans nuvA/uvsH*	Goldman and Kafer, 2004
	UBC13	Ubiquitin conjugating enzyme (E2), heterodimer with Mms2	*mus-46*	AN8702.3[a]	
	MMS2	Ubiquitin conjugating enzyme (E2), heterodimer with Ubc13	*mus-47*	AN3644.3[a]	
	REV3	Subunit of DNA polymerase zeta	*upr-1*	*A. nidulans uvsI*	Goldman and Kafer, 2004
	REV7	Subunit of DNA polymerase zeta	*mus-26*	AN2511.3[a]	
	REV1	Deoxycytidyl transferase	*mus-42*	AN3909.3[a]	
Recombination repair	*MRE11*	Subunit of MRX complex	*mus-23*	*A. nidulans mreA*	Goldman and Kafer, 2004
	RAD50	Subunit of MRX complex	*uvs-6*	*A. nidulans sldI*	Malavazi et al., 2005
	XRS2	Subunit of MRX complex	*mus-45*	*A. nidulans scaA*	Goldman and Kafer, 2004
HR	*RAD51*	Strand exchange protein, forms a helical filament with DNA	*mei-3*	*A. nidulans uvsC*	Goldman and Kafer, 2004
	RAD52	Anneals complementary single-stranded DNA	*mus-11*	*A. nidulans radC*	Goldman and Kafer, 2004
	RAD54	DNA-dependent ATPase	*mus-25*	AN10677.3[a]	
	RAD55	Stimulates strand exchange	*mus-48*	AN6728.3[a]	
	RAD57	Stimulates strand exchange	*mus-49*	AN10145.3[a]	
NHEJ	*YKU70*	Subunit of the Ku complex	*mus-51*	*A. nidulans nkuA*	Nayak et al., 2006
	YKU80	Subunit of the Ku complex	*mus-52*	*M. grisea Mgku80*	Villalba et al., 2008
	LIG4	DNA ligase required for NHEJ	*mus-53*	*A. oryzae ligD*	Mizutani et al., 2008
	LIF1	Mammalian XRCC4 protein	*mus-56*	?	
	NEJ1	Mammalian XLF protein	*mus-57*	?	
DNA damage checkpoint	*MEC1*	Phosphatidylinositol 3 kinase, ATR homolog	*mus-9*	*A. nidulans uvsB*	Goldman and Kafer, 2004
	LCD1	Interact physically with Mec1p	*uvs-3*	*A. nidulans uvsD*	Goldman and Kafer, 2004
	TEL1	Phosphatidylinositol 3 kinase, ATM homolog	*mus-21*	*A. nidulans atmA*	Malavazi et al., 2006
Others	*SGS1*	3′-5′ DNA helicase	*mus-19/ qde-3*	*A. nidulans musN*	Goldman and Kafer, 2004
			recQ2	*AN443.3[a]	
	SRS2	3′-5′ DNA helicase	*mus-50*	AN3797.3[a]	
	MMS1?	S-phase-specific recombination-promoting factors.	*mus-7*	?	
	RTT109	Acetylation of histone H3 on lysine 56 (H3K56)	*mus-16*	AN8807.3[a]	

[a]Genes identified by annotation in *A. nidulans*.

[b]*S. pombe* gene.

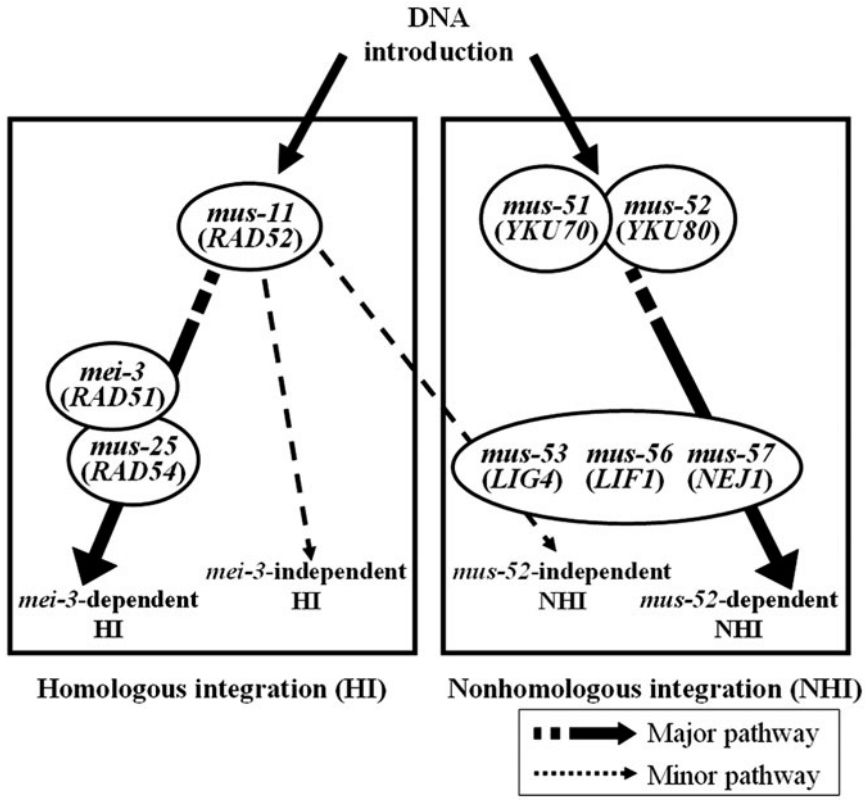

FIGURE 1 Pathways (major, thick arrows; minor, narrow dashed arrows) for integration of exogenous DNA into chromosomal DNA. Exogenous DNA is integrated into the chromosomes by two major pathways, *mus-11* dependent and *mus-52* dependent. The *mus-11*-dependent pathway has three branches. Two branches yield homologous integration (HI); one is *mei-3* dependent, and the other is *mei-3* independent. The third *mus-11* branch is *mus-52* independent but, like the major *mus-52*-dependent pathway, leads to nonhomologous integration (NHI). Both *mus-52*-dependent and *mus-52*-independent NHI pathways require *mus-53*, *mus-56*, and *mus-57*. The genes in parentheses represent *S. cerevisiae* homologs.

are used as the transformation host, homologous integration almost never occurs (Handa et al., 2000; Ishibashi et al., 2006). It seems likely, therefore, that these genes function in HR in *N. crassa*, as in *S. cerevisiae*.

NHEJ

NHEJ is the major pathway for repair of DSB in higher organisms, but among mutants showing high sensitivity to MMS or IR, NHEJ-defective mutants have not been found in fungi, suggesting the possibility of a backup system to NHEJ as in NER. However, NHEJ homologs of the human *KU70*, *KU80*, and *LIGIV* genes (*mus-51*, *mus-52*, and *mus-53*, respectively) have been identified in the *N. crassa* genome (Ninomiya et al., 2004; Ishibashi et al., 2006), and these mutants, like *mei-3* and *mus-25*, show significant sensitivity to MMS and bleomycin and slight sensitivity to UV.

When transforming DNA fragments share more than 1 kb of homologous sequence with the host genome, all transformants obtained in *mus-51*, *mus-52*, and *mus-53* mutants result from homologous integration, confirming that *mus-51*, *mus-52*, and *mus-53* function in NHEJ. This observation suggested a use for NHEJ-deficient mutant strains as hosts for efficient gene targeting/replacement experiments (Ninomiya et al., 2004; Ishibashi et al., 2006). This approach has also been successful with many other fungi (*A. nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Magnaporthe grisea*, *Podospora anserina*, *Cryptococcus neoformans*, *Cryphonectria parasitica*, and *Candida glabrata*) and has been used for gene improvement and high-throughput gene knockout projects in fungi (Colot et al., 2006).

Transformation in the *N. crassa mei-3 mus-52* double mutant produces very few transformants, but all result from homologous integration, indicating the existence of a *mei-3*-independent minor homologous integration pathway (Ishibashi et al., 2006). Another minor pathway may be microhomology-mediated end joining (Fig. 1).

Regulation of Recombination Repair in *Neurospora*

mus-23, *uvs-6*, and *mus-45* mutants are highly sensitive to a range of mutagens, including MMS, IR, UV, histidine, HU, and 4-nitroquinoline 1-oxide (4NQO), and appear to be homologs of *S. cerevisiae MRE11*, *RAD50*, and *NBS1/XRS2*, respectively (Watanabe et al., 1997; Borkovich et al., 2004). Each of these mutants and all double-mutant combinations have the same sensitivity to mutagens; homozygous crosses are sterile, and a characteristic "stop-start growth" phenotype is also observed. This suggests that these three proteins form a complex, designated MRX/MRN. Double mutants carrying *mei-3* (HR) and *mus-51*, *mus-52*, or *mus-53* (NHEJ) showed similar sensitivity to that of *uvs-6* (*S. cerevisiae*

RAD50 homolog), and double mutants of *uvs-6* with *mei-3* (HR group) or *mus-51* (NHEJ group) also showed sensitivity similar to that of *uvs-6*. These results indicate that *uvs-6* is epistatic to *mei-3* and *mus-51* in MMS sensitivity, and since DSB are repaired by either HR or NHEJ, the UVS-6/MUS-23/MUS-45 complex regulates a process upstream of both recombination repair pathways (Fig. 1). Interestingly, mutation of *uvs-3* (described below) in any mutant of this group results in a synthetic lethal.

Checkpoint Control

Checkpoint control enables cells to coordinate DNA repair and the cell cycle following damage. In *Homo sapiens*, there are two interacting systems, ATR and ATM, that are involved in complex phosphorylation cascades. Mutants defective in checkpoint control are extremely sensitive to a range of mutagens. The *N. crassa uvs-3* mutant is sensitive to UV, IR, MMS, mitomycin C, and HU; homozygous crosses are barren, and hyphal growth shows a stop-start growth pattern (Schroeder, 1970; Newmeyer and Galeazzi, 1978). The spontaneous mutation rate is high but is not increased by mutagens (de Serres et al., 1980; de Serres, 1980; Inoue et al., 1981), and micronuclei are observed at high frequency (Kazama et al., 2008). The sequence of *uvs-3* suggests that it is a homolog of the human *ATRIP*, which functions in ATR checkpoint control (Kazama et al., 2008). Homologs are found in many other organisms, including Lcd1 in *S. cerevisiae*, Rad26 in *Schizosaccharomyces pombe*, and UvsD in *A. nidulans*. Phosphorylation of ATRIP is dependent on ATR. In the same epistasis group as *uvs-3*, the *N. crassa mus-9* gene is an ATR homolog and has a phenotype very similar to that of *uvs-3* mutants: high sensitivity to mutagens, genome instability, deficiency in induced mutagenesis, and a meiotic defect (Kazama et al., 2008). ATR homologs, such as Mec1 in *S. cerevisiae*, Rad3 in *S. pombe*, and UvsB in *A. nidulans*, are involved in cell cycle arrest, although only *MEC1* is essential for the process. *A. nidulans* and *N. crassa* ATR mutants are sterile in homozygous crosses, but *S. pombe rad3* mutants generate viable spores. A mutation in *mus-21*, a homolog of *S. cerevisiae ATM/TEL1*, is epistatic to *uvs-6* (an MRX member), and a double mutant with *uvs-3* or *mus-9* (ATR) is a synthetic lethal (Fig. 2). These relationships indicate that recombination repair is integrated into the phosphorylation cascade of *mus-21/ATM* damage checkpoint control. Further study of checkpoint mechanisms in fungi requires identification and analysis of other checkpoint-related genes such as Chk1, Chk2, and Rad53.

Others: MMS-Sensitive Mutants with Unusual Phenotypes

qde-3/mus-19

The MMS-sensitive *mus-19* mutant is an allele of the quelling-defective mutant *qde-3*. This gene is a member of the RecQ helicase family (3′-to-5′ DNA helicase) and is epistatic to *mei-3* and *uvs-2*, so *qde-3* has a function in both HR and PRR (Kato et al., 2004). *N. crassa* has two RecQ helicases, *qde-3* and *recQ2* (Pickard et al., 2003). QDE-3 is similar to *S. cerevisiae* Sgs1 and human BLM, while RecQ2 is similar to human RecQL5α. The *qde-3* mutant is sensitive to MMS, *N*-methyl *N*′-nitro-*N*-nitrosoguanidine (MNNG), camptothecin (CPT), and HU, while *recQ2* is much less sensitive. However, the *qde-3 recQ2* double mutant is more sensitive to MMS, MNNG, CPT, and HU than either single mutant, indicating that both RECQ homologs function in repair (Kato and Inoue, 2006). In homozygous crosses, *qde-3*, but not

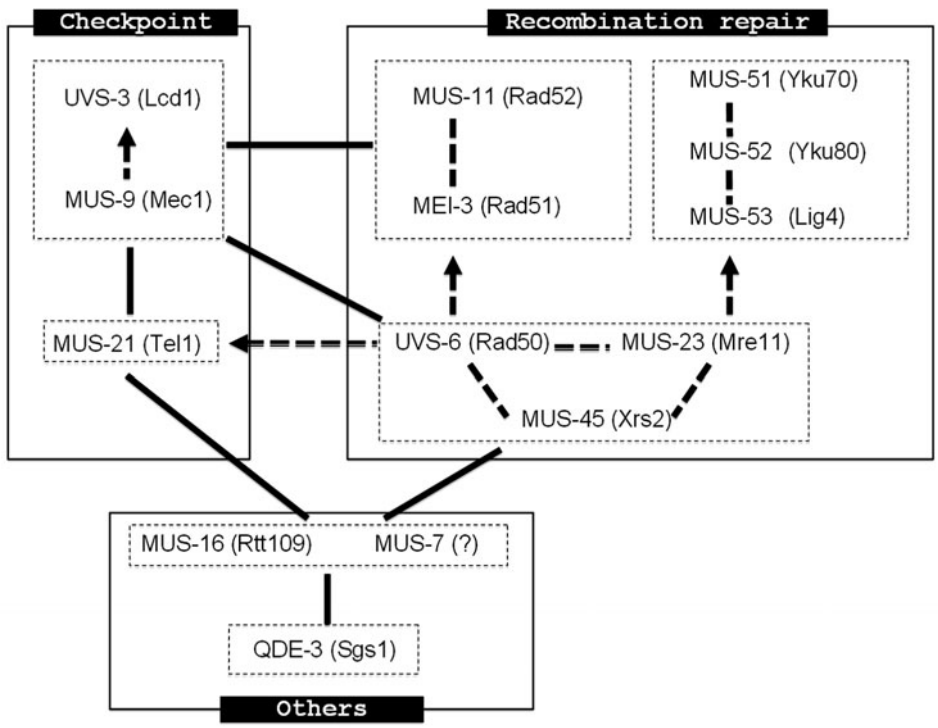

FIGURE 2 Epistatic and synthetic lethal relationships of mutagen-sensitive mutations in *N. crassa*. A solid line indicates a synthetic lethal relationship, and a dotted line indicates an epistatic relationship. Arrows indicate which mutation is epistatic.

recQ2, is sterile. *qde-3* has a high spontaneous mutation rate, as does the double mutant. Also, colony morphology is irregular and growth is unstable in the double mutant. The growth defect and the high spontaneous mutation rate of the double mutant are respectively suppressed by mutation in *mei-3* and *mus-52* (Kato and Inoue, 2006), suggesting that both RecQ homologs work downstream of *mei-3* in homologous recombination repair to maintain genome stability in *N. crassa*.

As noted above, *N. crassa mus-50* encodes a homolog of a 3′-to-5′ helicase, Srs2, and the *mus-50 qde-3 recQ2* triple mutant is lethal, indicating that at least one 3′-to-5′ helicase is necessary for survival (Suzuki et al., 2005). This lethality is suppressed by mutation in *mei-3*, *mus-11*, or *mus-25* (a homolog of *S. cerevisiae RAD54*). Genetic analysis shows that there are two independent pathways to repair strand breaks: one is dependent on QDE-3 and MUS-50, and the other is dependent on MUS-25 and RECQ2. Although there is no Srs2 homolog in the human genome, there are five RecQ homologs, each with an independent function. One behaves like Srs2 in *S. cerevisiae*, suggesting that during evolution, Srs2 has been functionally replaced by a duplicated RecQ homolog. Unlike *S. cerevisiae*, which has a single copy of each gene, *N. crassa* has two RecQ homologs and one Srs2 homolog, so filamentous fungi may be at an interesting stage in evolution.

mus-7 and *mus-16*

Mutants *mus-7* and *mus-16* are highly sensitive to MMS, ethyl methanesulfonate, 4NQO, HU, and CPT but less sensitive to UV, IR, and mitomycin C. DNA damaged by MMS generates DSB during DNA replication, and since IR also causes DSB, most mutants sensitive to IR also show high sensitivity to MMS. Thus, the sensitivity phenotype of *mus-7* and *mus-16* mutants is novel (Kafer, 1981; Inoue and Schroeder, 1988). These mutants are sterile in homozygous crosses, in contrast to mutants defective in base excision repair that remove alkylated bases generated by MMS. Epistasis analyses indicate that *mus-7* and *mus-16* mutants do not belong in any of the three major repair groups or in damage checkpoint control. MMS induces mutation at high frequency in both mutants, which also show chromosomal instability. The *mus-7* gene encodes a protein with no known motifs to provide clues to its function. A homolog in *S. pombe*, *mus-7*, has a similar phenotype (Yokoyama et al., 2007). Interestingly, the CPT sensitivity of *mus7* was suppressed by mutation in *top1* (topoisomerase 1). On the other hand, double mutants carrying *mus7* and *rqh1* (RecQ family gene defective) show higher sensitivity to MMS and CPT than either single mutant (Yokoyama et al., 2007). In *N. crassa*, the equivalent double mutant is lethal (Fig. 2). Although it has been reported that *S. cerevisiae MMS1* has no homolog in other organisms, it appears to be a functional homolog of *S. pombe* Mus7 and *N. crassa* MUS-7 (Hryciw et al., 2002). Like *N. crassa mus-7*, in comparison with the wild type, the *S. cerevisiae mms1* mutant is highly sensitive to killing by MMS or CPT and mildly sensitive to UV but no more sensitive to IR. *rad52* is epistatic to *mms1* for MMS and CPT sensitivity in *S. cerevisiae*, and the *mms1* mutant shows slow growth, abnormal morphology, and genomic instability. The *N. crassa mus-7 mus-11* double mutant grows more poorly with time (M. Yokoyama, A. Inamori, C. Ishii, S. Hatekeyama, and H. Inoue, unpublished data). Mus-7 is involved in replication-associated damage repair, and because of the lack of homology to other known repair proteins, further analysis may open a new area in research into DNA repair in addition to the known major repair pathways.

The *mus-16* mutation is lethal in combination with either *mus-21*, *mus-23*, or *qde-3*, and *mus-7* is epistatic to *mus-16* (Fig. 2), suggesting that both are in the same repair group. *N. crassa mus-16* is defective in acetylation of Lys56 of H3 (Yokoyama et al., unpublished), like the *S. cerevisiae* homolog Rtt109 (Schneider et al., 2006; Driscoll et al., 2007), indicating that some mutants defective in chromatin maintenance and modification also have a mutagen-sensitive phenotype. Such mutants may be useful for analysis of chromatin dynamics.

RECOMBINATION

Linkage and Crossing-Over

As far back as 1905, Bateson, Saunders, and Punnet demonstrated that some sweet pea genes were typically inherited together but sometimes segregated independently (Bateson et al., 1905). Subsequently, Morgan (1910, 1911) demonstrated that the degree of linkage of genes on the *D. melanogaster* X chromosome varied between different gene pairs. Linkage was assumed to reflect (Morgan, 1911) the likelihood that a chromosomal exchange, or crossover (Morgan and Cattell, 1912), would occur between genes and thus could be used as a measure of the distance separating that gene pair. Sturtevant (1913) used the relative frequency of crossovers between loci to plot the relative positions of six genes on a map of the *Drosophila* X chromosome, creating the first linkage map.

Creighton and McClintock (1931) demonstrated a correlation, predicted some 20 years earlier by Janssens (1909), between the number of chiasmata, visible connections between homologous chromosomes during meiosis, and the number of crossovers in *Zea mays*. Indeed, it has since been demonstrated that in most organisms, normal meiotic chromosome segregation is dependent on the occurrence of at least one such crossover in each bivalent (Baker et al., 1976).

Gene Conversion

One feature of the Ascomycetes that makes them ideal subjects for recombination studies is that all products from a single meiosis are retained in the ascus. Indeed, it was an ascomycete that provided the first indication that recombination was not always reciprocal. Lindegren (1953) found that segregation of mutations in *S. cerevisiae* did not always conform to the expected Mendelian ratio. Some asci contained three wild-type and one mutant or one wild-type and three mutant spores (6:2 and 2:6, respectively, in an eight-spored ascus) instead of the expected two wild-type and two mutant spores. This phenomenon was called "gene conversion," and although initially thought to be due to a rare and peculiar chromosomal aberration, it was soon found to be common among Ascomycetes.

Mary Mitchell (1955) demonstrated that wild-type progeny arising from crosses between two *pdx* mutant strains of *N. crassa* did not result from abnormal segregation of entire chromosomes but from changes in a short chromosomal region, since markers flanking the *pdx* locus segregated normally. Mitchell demonstrated that this effect was not due to crossing-over, as no reciprocal double mutants were found in asci containing wild-type progeny. While the effect was not due to a crossover, crossing-over between *pdx* and a marker nearby was elevated in asci showing conversion. This was also observed at other loci in *Neurospora* (Case and Giles, 1958a, 1958b; Stadler, 1958; Murray, 1960) and *A. nidulans* (Pritchard, 1955). Gene conversion has also been demonstrated in *S. fimicola*

(Olive, 1959) and *A. immersus* (Lissouba et al., 1962). In *Sordaria*, as in *Neurospora*, gene conversion is associated with a local increase in crossing-over (Kitani et al., 1962).

PMS

Olive (1959) found, in addition to 6:2 and 2:6 ratios, also 5:3 and 3:5 ratios of spore color segregation in asci from *Sordaria*. Moreover, an additional class of abnormal asci, aberrant 4:4, was found in the same species (Kitani et al., 1962). Here, pairs of sister spores, which arose from a postmeiotic mitosis and thus should be genetically indistinguishable for spore color, were different. Segregation of the aberrant allele was not due to abnormal behavior of an entire chromosome, since the sister spores had identical flanking markers. Thus, the spore color mutation must have segregated during the mitotic division that follows meiosis, implying that the recombinant chromosome from which the spore pair derived was a heteroduplex (hDNA), a DNA molecule in which the two strands were not fully complementary. This is an example of postmeiotic segregation (PMS), which has also been observed in *S. cerevisiae* (Fogel et al., 1979) and in *N. crassa* (Case and Giles, 1964).

Models of Meiotic Recombination

The segregation patterns of parental DNA in recombinant chromosomes from yeasts and filamentous fungi as considered above stimulated Whitehouse (1963) and Holliday (1964) to formulate models of meiotic recombination, and aspects of both models remain valid to this day. While subsequent theoreticians built upon the foundations laid by Whitehouse and Holliday, common to each model was a single mechanism to account for all HR. However, it has recently become clear that this assumption was erroneous and that recombination, while thought to have a single mode of initiation, proceeds by at least three distinct pathways (reviewed by Baudat and de Massy, 2007; Cromie and Smith, 2007; and Lynn et al., 2007). Moreover, the relative contribution of each pathway to the total recombination quota varies from organism to organism.

Until recently it was thought that the DSB repair (DSBR) model (Szostak et al., 1983; Sun et al., 1991) could explain all recombination. In this model, a break in both strands of one homolog (Fig. 3B) is resected to generate single strands. Invasion of the homologous duplex by a 3′ end (Fig. 3C) and DNA synthesis, using the complementary

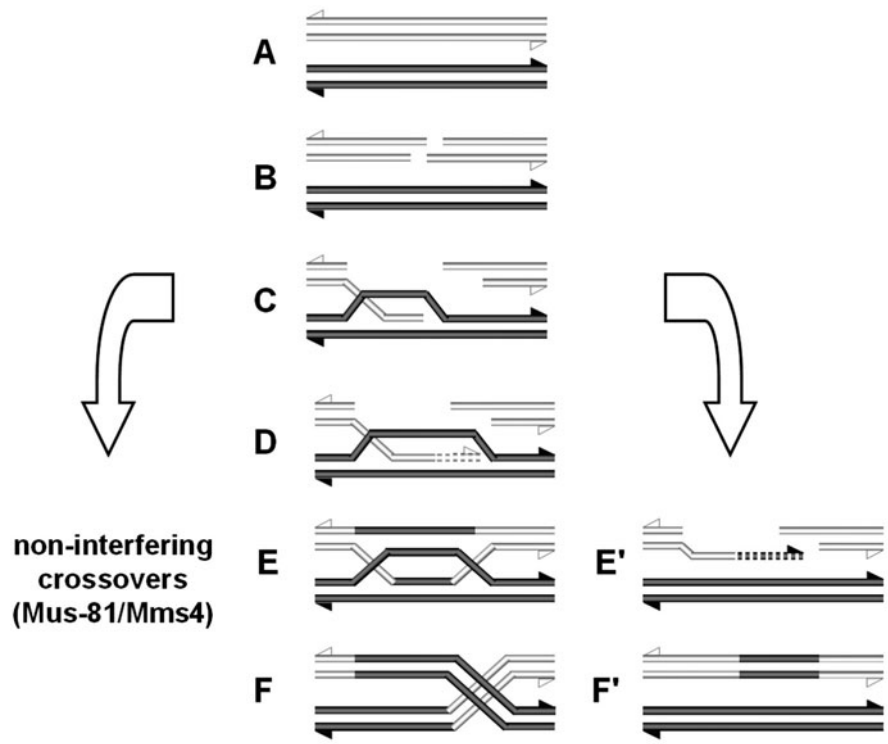

FIGURE 3 Models for meiotic recombination (after Baudat and de Massy, 2007). The central column depicts the generation of crossovers. All recombination is thought to be initiated by a break in both strands of the duplex (B). The 5′ ends are resected, and one of the 3′ ends invades the homologous duplex (C), where it is extended by DNA polymerase using the homolog as a template (D). The D loop formed by displacement of a strand of the invaded chromosome provides a template for repair of the second strand of the initiating chromosome (E). The ligation of ends forms two Holliday junctions that when resolved yield a crossover (F). Synthesis-dependent strand annealing is depicted on the right branch. Here, instead of ligation leading to paired Holliday junctions, the newly synthesized DNA ends are unraveled from the template to anneal one with the other (E′). The gap is closed and then ligated (F′). Only the initiating chromosome is converted, and there is no crossing-over. The left branch indicates the existence of a pathway yielding noninterfering crossovers, although details of the mechanism remain to be elucidated.

strand of the intact duplex as template, leads to the formation of a D loop that provides a template for repair synthesis of the second 3′ end (Fig. 3D). Ligation of the free ends produces paired Holliday junctions (Fig. 3E). Branch migration can give symmetric heteroduplex, and resolution of the two Holliday junctions in the same or opposite sense yields crossover (Fig. 3F) or noncrossover (Fig. 3F′) outcomes, each associated with conversion resulting from correction of mismatched bases in hDNA. The DSBR model explains the higher probability of conversion of the initiating chromosome but also suggests that one-half of the chromosomes with a conversion event should also have a crossover near the converted site, although most mitotic conversions (Pâques and Haber, 1999) and some meiotic conversions (Bowring and Catcheside, 1996) yield few associated crossovers. Moreover, since conversion events with and without a crossover are alternative outcomes of the resolution event, this model predicts that they should occur at the same time, but they do not (Allers and Lichten, 2001). These discrepancies led to the formulation of the synthesis-dependent strand annealing (SDSA) model.

The SDSA model (Nassif et al., 1994; Ferguson and Holloman, 1996; Pâques et al., 1998) accounts for the predominance of conversion of the initiating chromosome, a variable degree of association of conversion with crossing-over, and the differential timing of crossover and noncrossover events. In the current version of the SDSA model, following extension by repair synthesis, the invading strand is reannealed with the initiating duplex (Fig. 3E′) and no Holliday junction is formed, a circumstance that leads only to conversion of the initiating chromosome and no crossover. The finding that replication errors occurring during repair synthesis usually appear only on the initiating chromosome provides support for the SDSA model (Strathern et al., 1995; Pâques et al., 1998).

More recently, a third recombination pathway, which requires Mus81 and Mms4 and generates crossovers independently of the DSBR route, has been identified. The majority of crossovers in *S. cerevisiae* require the "ZMM proteins," comprising Zip1, Zip2, Zip3, Zip4/Spo22, Mer3, Msh4, and Msh5 (Borner et al., 2004). ZMM-dependent crossovers exhibit crossover interference (Sturtevant, 1913), the process that reduces the chance that crossovers will occur close together. *S. cerevisiae* ZMM mutants exhibit greatly reduced levels of crossing-over, and the residual Mus81/Mms4-dependent crossovers show no interference (de los Santos et al., 2003). Interestingly, all crossovers in *S. pombe* are Mus81/Mms4 dependent (Smith et al., 2003) and also lack interference (Munz, 1994). Crossovers in *A. nidulans*, which lack interference (Strickland, 1958), may also result from the Mus81/Mms4 pathway.

Initiation of Recombination

It is thought that all meiotic recombination in *S. cerevisiae* is initiated by SPO11-generated DSB (Keeney and Neale, 2006). SPO11-catalyzed DSB are also responsible for initiation of meiotic recombination in *Coprinus cinereus* (Celerin et al., 2000), *Sordaria macrospora* (Storlazzi et al., 2003), and *N. crassa* (Bowring et al., 2006). However, studies using filamentous fungi provide just a hint that SPO11 might not be required for the initiation of all recombination events. In *S. macrospora*, for example, knocking out *SPO11* reduces crossing-over about 10-fold across a large interval on chromosome 4 but does not abolish it (Storlazzi et al., 2003). In *N. crassa*, mutation of *spo11* seems to exhibit a variable effect on recombination, depending upon where in the genome one samples. Crossing-over is severely reduced in an interval on LG VII in an *N. crassa spo11* mutant, but at *his-3* on LG I there is no apparent reduction in either gene conversion or crossover frequency in homozygous pairings of three different *spo11* mutants. Since this lack of effect occurs at the target region of *rec-2*, now known to be an activator of recombination (see below), this raises the possibility that initiation here is independent of SPO11. However, since pairings of *N. crassa spo11* mutants exhibit an increased frequency of hyperploid spores, it is conceivable that the observed crossing-over occurs after meiosis in these partially diploid nuclei, and normal meiotic recombination is initiated exclusively by SPO11 (Bowring et al., 2006).

Polarity and Recombination Hot Spots in Filamentous Fungi

Polarity of conversion, a linear relationship between conversion frequency at a given site and its position in the locus, was first observed by Lissouba and Rizet (1960), working with a series of allelic spore color mutants in *A. immersus*, but has also been noted in *A. nidulans* (Siddiqi, 1962) and in *N. crassa* (Case and Giles, 1958a; Murray, 1963; Stadler and Towe, 1963). Shortly afterwards, conversion at *ARG4* of *S. cerevisiae* was shown to involve a tract of DNA sequence; if a site at the low end of the conversion gradient is converted, other sites towards the high end of the gradient are often converted along with it (Fogel and Mortimer, 1970).

Use of molecular techniques such as PCR and DNA sequencing have enabled analysis of recombination at the molecular level in *N. crassa*. The lesions defining *his-3* mutant alleles K1201, K504, K458, K874, K26, and K480 have been identified (Yeadon and Catcheside, 1999; Yeadon et al., 2001, 2002; Rasmussen et al., 2002), and the order of these sites on the physical map is the same as on the genetic map (Angel et al., 1970), confirming that polarity reflects relative physical position along a chromosome. Polarity indicates that recombination starts in a particular place and proceeds in a linear fashion. Supportive of this, studies with *N. crassa* using a large chromosomal inversion (Murray, 1968) showed that polarity is dependent on sequences near the gene within which recombination occurs, so recombination is presumably initiated within these sequences at a recombination initiation site, or hot spot.

It is likely that many recombination hot spots exist in *N. crassa*, as both allelic recombination and crossing-over occur at frequencies that vary with location in the genome. For example, gene conversion has been detected at high frequency at the *cot-1* and *lys-4* loci (P. J. Yeadon, F. J. Bowring, and D. E. A. Catcheside, unpublished data), and recombination is known to be an uncommon event in the vicinity of *am* (Bowring and Catcheside, 1998). The only well-characterized hot spot, however, is *cog*, centromere distal of the *his-3* locus (Bowring and Catcheside, 1991; Yeadon and Catcheside, 1995, 1998), although a similar site may exist in *A. immersus*.

ccf-2 in *Ascobolus*

The *A. immersus* conversion control factor *ccf-2* acts in *cis* at the adjacent *wI* locus (Lamb and Helmi, 1978; Helmi and Lamb, 1983). Three alleles are known, P, 91, and K; and crosses homozygous for P yield the highest gene conversion frequency in *wI*. The 91 and K alleles appear to act as partially dominant suppressors of recombination at *wI* (Lamb and Helmi, 1978). This factor appears to behave in a fashion similar to that of the *N. crassa* hot spot *cog*.

cog

cog has alleles with two phenotypes, high (cog^+) and low (*cog*) frequency recombination (Angel et al., 1970), influencing both allelic recombination in *his-3* and crossing-over between *his-3* and the centromere-distal gene, *ad-3* (Catcheside and Angel, 1974). Although cog^+ was previously thought to be completely dominant to *cog*, the alleles are actually codominant (Yeadon et al., 2004a). The genetic interval between *his-3* and *ad-3* is 15.5 centimorgans (cM) when cog^+ is homozygous, 7.5 cM when cog^+ is heterozygous, and only 1.4 cM if *cog* is homozygous. Likewise, two copies of cog^+ in a *his-3* heteroallelic cross yield approximately twice as many His^+ recombinants as a single copy in an otherwise identical cross. Thus, the *cog* alleles in a cross appear to operate independently of one another to initiate recombination events.

Sequences within the 900-bp *cog* region, defined by crossover mapping (Yeadon and Catcheside, 1995), show multiple strain-specific differences. However, the *cog* variant first found in Lindegren Y8743 (Angel et al., 1970) is the only naturally occurring cog^+ allele known (Yeadon and Catcheside, 1995, 1999), and *cog* variants present in Lindegren *A*, Emerson *A*, and St Lawrence 74*A* strains all appear to have the same low-frequency recombination and thus *cog* phenotype. Conservation of much of the *cog* region is known to be irrelevant, but a 10-bp sequence, ~3.4 kb from *his-3*, including two single-nucleotide polymorphisms (SNPs) is definitely required for the high-frequency recombination phenotype (Yeadon and Catcheside, 1998). Deletion of the entire *cog* region, with the exception of 60 bp in which the 10-bp sequence lies, does not substantially alter the cog^+ phenotype (A. Romsang, P. J. Yeadon, F. J. Bowring, and D. E. A. Catcheside, unpublished data), so this short motif may be sufficient for high-frequency recombination, at least at this chromosomal location.

N. crassa strains have been constructed in which 1.8 kb of native sequence between *his-3* and *cog* is replaced with a series of foreign sequences (Yeadon et al., 2002; Rasmussen et al., 2002), including the human *Herpes simplex thymidine kinase* (*TK*) gene. Analysis of recombinant progeny of *TK* heterozygotes indicates that recombination is initiated distal to the replacement region (Yeadon et al., 2001), which is located 1.2 kb proximal to the SNP motif required for high-frequency recombination. In addition, a peak in conversion close to the motif (Yeadon and Catcheside, 1998) suggests that the initiation site is at, or close to, the SNP motif (Yeadon et al., 2004b).

The series of insertion/replacement strains has been used to analyze recombination in crosses in which the distance between *cog* and *his-3* varies from 1.7 kb to nearly 6 kb. The frequency of His^+ progeny from heteroallelic crosses increases exponentially as the distance from *cog* declines (Yeadon et al., 2002), suggesting that recombination events are initiated at the same place and terminate in a stochastic fashion, one possible explanation for polarity. In organisms as diverse as *Drosophila* (Hilliker et al., 1994) and *S. pombe* (Grimm et al., 1994), a similar relationship between the distance separating markers and the chance of coconversion has been observed, so the distance that a recombination event travels may be a stochastic process in most organisms.

Data from both *S. cerevisiae* (Nicolas et al., 1989) and *S. pombe* (Gutz, 1971) suggest that the chromosome carrying the more active hot spot, on which conversion is more likely to be initiated, is usually the recipient of information. *N. crassa* is no exception, for the chromosome bearing cog^+ is almost always the one that is converted (Catcheside and Angel, 1974; Yeadon and Catcheside, 1998; Yeadon et al., 2001). This relationship is not simple, however, as conversion tracts in *N. crassa* can be very complex. Between *his-3* and *cog*, the sequences from different wild-type strains diverge by up to 3.4% (Yeadon and Catcheside, 1999), thus providing many silent markers for analysis of recombinant progeny. Use of these markers has shown that many conversion tracts at *his-3* are discontinuous, with patches of sequence from each parent (Yeadon and Catcheside, 1998), and similar although less complex events have been recorded at *am* (Bowring and Catcheside, 1998).

A Template-Switching Model for Recombination

TM429 is a *his-3* mutant generated in a cog^+ strain, in which the mutant allele is a translocation that separates the proximal end of *his-3* from *cog* (Catcheside and Angel, 1974). In a translocation heterozygote, therefore, a *his-3* allele proximal to TM429 lies on the opposite side of the breakpoint from *cog*. The frequency of His^+ spores from such a cross is low and unregulated by *rec-2* (see below), unless the intact chromosome also carries cog^+, so recombination can cross the translocation heterology provided it is initiated on the unbroken chromosome (Catcheside and Angel, 1974). Supportive of this, molecular analysis of His^+ progeny of a TM429 heterozygote showed that conversion can occur on both sides of the translocation breakpoint in individual progeny (Yeadon et al., 2001). It has also been observed that conversion tracts are often patchy in His^+ progeny of non-translocation homozygotes (Yeadon and Catcheside, 1998), even in the absence of mismatch repair (Koh and Catcheside, 2007). This suggests that if recombination is initiated by a DBS close to cog^+, the break is repaired by DNA synthesis that can use either the homologous chromosome or the initiating chromosome as a template and may switch from one to the other during repair, a template-switching variant of the SDSA model (Pâques and Haber, 1999; Yeadon et al., 2001). Like other SDSA models, a crossover can arise by resolution of single (Ferguson and Holloman, 1996) or double Holliday junctions (Pâques et al., 1998).

The Role of MMR in Recombination

Sequences that differ in the chromosomes involved in a recombination event, such as point mutations, insertions, and deletions, yield hDNA with mismatched bases (Modrich, 1991; Strand et al., 1993). The mismatch repair (MMR) pathway recognizes these and corrects them with variable degrees of efficiency, which may generate novel sequence combinations.

MMR in bacteria utilizes the MutS, MutL, and MutH proteins. MMR is initiated when a dimer of MutSp binds to a mismatch (Su et al., 1988), followed by the binding of MutLp dimer to the MutS-mismatch complex, increasing the stability of the complex (Grilley et al., 1989; Mankovich et al., 1989) and activating MutH endonuclease (Grafstrom and Hoess, 1987), which cleaves at the GATC site (Au et al., 1992). Since MutH cleavage is in the unmethylated strand, repair is targeted to the newly synthesized strand.

In *S. cerevisiae*, six *MutS* (*MSH1* through *MSH6*) and four *MutL* (*PMS1* and *MLH1 through MLH3*) homologs have been identified (reviewed by Pâques and Haber, 1999, and Borts et al., 2000). A heterodimer of MSH proteins recognizes the mismatch and an MLH protein heterodimer binds to the mismatch/MSH complex. The MSH and MLH proteins involved in each dimer depend on the nature of the mismatch, with MSH2/MSH3 recognizing small loops, insertions, and deletions, which are repaired with

the assistance of PMS1/MLH2, and single-base frameshift mutations, which utilize MLH1/MLH2 for repair. The MSH2/MSH6 heterodimer binds to small loops and single-base mismatches and recruits PMS1/MLH1 (Borts et al., 2000). Thus, all Mshp heterodimers involved in MMR include Msh2p, but only two of the three Mlhp repair heterodimers include Pms1p.

Mutations in *MSH2* and *PMS1* in *S. cerevisiae* increase PMS, as mismatches in hDNA remain uncorrected, and also have a mutator phenotype, increasing microsatellite instability and spontaneous mutation up to 1,000-fold (Strand et al., 1993). In addition, spores from MMR-deficient diploids show reduced viability compared to that of spores from wild-type crosses, to 60 to 80% in *S. cerevisiae* (Williamson et al., 1985) and 86% in *S. pombe* (Rudolph et al., 1999). Also in *S. pombe*, a *msh2*Δ (deletion) mutant exhibits abnormal meiotic chromosome structures, with linear elements frequently aggregated, a rare occurrence in the wild type (Rudolph et al., 1999).

N. crassa also has six *MutS* and four *MutL* homologs (Borkovich et al., 2004). Strains defective in *msh-2* (Koh and Catcheside, 2007) and *pms1* (L. Y. Koh and D. E. A. Catcheside, unpublished data) have been generated by repeat-induced point mutation (Selker, 1990), and subsequent *msh-2* deletion mutants have been made using the split-marker technique of Catlett et al. (2003), with the coding sequence replaced by the hygromycin resistance gene.

Both *msh-2*^{RIP} and *pms1*^{RIP} strains have a mutator phenotype, as the mitotic reversion rate of the *his-3* K458 allele is increased 20-fold in *msh-2* and *pms1* mutants compared to wild-type strains (Koh and Catcheside, 2007; Koh and Catcheside, unpublished). Moreover, although 85 to 95% of the spores from wild-type *N. crassa* crosses are viable, *msh-2*^{RIP} and *pms1*^{RIP} homozygotes yield 20 to 30% inviable colorless spores and only 60 to 70% of pigmented spores are viable.

In an analysis of 42 *N. crassa* octads from *msh-2*Δ homozygotes, the only examples of non-Mendelian segregation of mutant alleles were PMS (5:3 or 3:5), confirming that Msh-2 is required for MMR in *N. crassa*. In addition, conversion tracts in His$^+$ progeny of *msh-2*^{RIP} homozygotes are at least as patchy as in progeny of wild-type crosses (Koh and Catcheside, 2007). Thus, interruptions to conversion tracts cannot be due solely to patchy MMR, providing support for the template-switching hypothesis of recombination (Yeadon et al., 2001).

Sequence heterology at levels commonly found in *N. crassa* crosses prevents crossing-over in *S. cerevisiae*, although absence of Msh2 restores exchanges in the heterologous interval (Chambers et al., 1996). However, even in *N. crassa* crosses between strains of different wild-type origin, crossing-over between *his-3* and *ad-3* is unaffected by *msh-2*Δ, although the *lys-4* to *mat* interval is increased up to twofold in some crosses (Yeadon et al., unpublished). Also, the frequency of His$^+$ allelic recombinant spores in the progeny of *N. crassa msh-2*Δ homozygotes is 150 to 160% of that of otherwise isogenic wild-type diploids, and this effect is unchanged by SNPs within the *his-3* gene (Yeadon et al., unpublished). It thus seems that Msh-2 does not regulate recombination in the *his-3* region, although the effect on the *lys-4* to *mat* interval suggests that crossing-over here may be regulated by Msh-2. Since Msh-2 does not regulate *his-3* allelic recombination, the increase in His$^+$ progeny in *msh-2*Δ homozygotes suggests that the direction in which hDNA within *his-3* is repaired by Msh-2 shows bias toward restoration of the original mutant allele rather than toward gene conversion.

The *pms1*^{RIP} homozygotes show no obvious recombination phenotype, although recombination in *his-3* may be slightly increased, and the double homozygote has the same phenotype as the *msh-2*^{RIP} homozygote (Koh and Catcheside, unpublished), suggesting that, as in *S. cerevisiae*, Msh-2 and Pms-1 operate in the same pathway.

Msh-4 and Crossing-Over in *Neurospora*

MSH4 and MSH5, members of the same protein family as MSH2, are not involved in MMR but rather have a role in crossing-over (reviewed by Hoffmann and Borts, 2004). MSH4 and MSH5 are known to form a heterodimer, and mutation in either has been observed to yield the same phenotype in many organisms: increase in chromosomal nondisjunction and a reduction in crossing-over (Ross-Macdonald and Roeder, 1994; Zalevsky et al., 1999; Hollingsworth et al., 1995; Hunter and Borts, 1997). In addition, MSH4 localizes at synapsis initiation sites during yeast and mammalian meiosis (Novak et al., 2001; Neyton et al., 2004), and chromosome synapsis in *S. cerevisiae msh4* mutants is delayed and defective (Novak et al., 2001). Although MSH4/MSH5 is absolutely required for crossing-over in *C. elegans* (Zalevsky et al., 1999), crossovers occur at a reduced frequency in *S. cerevisiae msh4* mutants. These MSH4-independent exchanges do not show normal crossover interference but are distributed at random in the intervals in which they fall.

An ortholog of the highly conserved *MSH5* has been identified in *N. crassa* (NCU09384.1) by bioinformatic analysis (Borkovich et al., 2004), and the most likely *msh-4* candidate has been deleted (Conway et al., 2006). Deletion of *msh-4* is dominant, due to meiotic silencing of unpaired DNA (MSUD) (Shiu and Metzenberg, 2002), and mutants exhibit defects in meiosis; sporogenesis is delayed, and fertility is reduced to 20% of that of the wild type (Conway et al., 2006). Although *msh4*Δ reduces recombination in the *mat* to *lys-4* and *lys-4* to *ad-3* intervals, residual recombination occurs at about 30% of the wild-type frequency (S. Conway, P. J. Yeadon, F. J. Bowring, and D. E. A. Catcheside, unpublished data), suggesting that a ZMM protein-independent crossover pathway operates in *N. crassa* as it does in *S. cerevisiae* (see above). Whether these remaining crossovers lack interference cannot be determined in the *his-3* region, as the high frequency of gene conversion at both *his-3* and *lys-4* precludes measurement of interference by random spore analysis, and *msh4*Δ mutants do not yield full octads, preventing tetrad analysis of crossover distribution.

REGULATION OF RECOMBINATION

A number of genes that modulate recombination frequency in distinct genomic regions have been discovered in the filamentous fungi. These include genes that promote or suppress recombination and that act either in their immediate vicinity or at one or more remote loci.

Ascobolus Conversion Control Factors (*ccf* and *cv*)

A. immersus carries a number of conversion control factors that regulate gene conversion frequency in specific genomic regions. These fall into three classes, of which the first class, *ccf-2*, has been described above.

ccf genes in the second class act at specific but remote loci and comprise *ccf-3* and *ccf-4* (Helmi and Lamb, 1983). The *ccf-3E* allele increases conversion frequency at *wI* and is dominant to the *ccf-3*e allele. The action of the *ccf-4R* allele depends on the *ccf-2* and *ccf-3* alleles in a cross. When

ccf-3E is present, *ccf-4R* reduces recombination in *wI* only in the absence of *ccf-2(91)* (Helmi and Lamb, 1983).

A third class of *Ascobolus ccf* genes suppresses recombination at a nearby locus only when heterozygous. Crosses heterozygous for different pairs of *ccf-5* and *ccf-1* alleles show reduced gene conversion rates at the *w9* and *w62* loci, respectively (Emerson and Yu-Sun, 1967; Howell and Lamb, 1984; Lamb and Shabbir, 2002). Similarly, gene conversion at the *b1*, *b2*, *b4*, and *b6* loci is reduced in *cv1*, *cv2*, *cv4*, or *cv6* heterozygotes, respectively, and in addition to this effect, crossing-over between the *b4* and *b6* loci is reduced in *cv4* and *cv6* heterozygotes (Girard and Rossignol, 1974). The molecular bases of these effects on recombination have not been explored.

ss in *Neurospora*

ss is analogous to the *ccf-1*, *ccf-5*, and *cv* elements of *Ascobolus*. Catcheside (1981) found that recombination between *nit-2* alleles is influenced by the wild-type origin of the parental strains, with crosses of homozygous origin yielding higher allelic recombination frequencies than those where the two strains are from different backgrounds. This heterozygous suppression of recombination is due to a genetic element called *ss*, which is tightly linked to *nit-2*. Recombination is suppressed 2- to 20-fold depending upon the *ss* alleles present, but unlike *cog*, each *ss* allele does not operate independently; crosses homozygous for any *ss* allele yield more Nit^+ spores than those in which the *ss* alleles are heterozygous.

Neurospora rec Genes

The three known *N. crassa rec* genes, *rec-1*, *rec-2*, and *rec-3*, act *in trans* at specific regions of the genome (Fig. 4). Each dominant *rec-n*$^+$ allele was thought to produce a diffusible product (Catcheside, 1977) that reduces recombination in its target regions by about 1 order of magnitude. The *rec-1* gene acts at *his-1* on linkage group V (Jessop and Catcheside, 1965) and at *nit-2* on LGI (Catcheside, 1970), but not at 10 other loci tested (Catcheside and Austin, 1969). The *rec-2* locus, on LGV (Smith, 1968), modulates recombination in two intervals on LGI, *his-3* to *ad-3* (Angel et al., 1970; Catcheside and Corcoran, 1973) and *sn* to *arg-3* (Catcheside and Corcoran, 1973), and between *his-5* and *pyr-3* on LG IV (Smith, 1966).

In *rec-2*$^+$ hetero- or homozygotes, recombination between *his-3* alleles is reduced 30-fold when *cog*$^+$ is present and 4-fold in crosses homozygous for *cog*, dropping the recombination rate for both *cog* genotypes to the same low level (Angel et al., 1970). Recombination appears to be initiated at the 5′ end of *his-3* in the presence of *rec-2*$^+$, rather than at *cog*, which lies several kilobases 3′ of *his-3* (Catcheside and Angel, 1974; Yeadon and Catcheside, 1998), suggesting that initiation of recombination at *cog* is suppressed by *rec-2*$^+$ (Catcheside and Angel, 1974).

rec-3 regulates recombination at *am* on LG V (Catcheside, 1966) and at *his-2* on LG I (Catcheside and Austin, 1971). There are three known alleles, *rec-3*, *rec-3*^{L}, and *rec-3*$^+$. The *rec-3*$^+$ allele is dominant to both *rec-3* and *rec-3*^{L} at *his-2*, but at *am*, the effect of *rec-3*^{L} on recombination is intermediate between that of *rec-3* and *rec-3*$^+$ (Catcheside, 1974).

While the dominant allele of each *N. crassa rec* gene appears to suppress recombination at the target, it now appears that this is an artifact of another meiotic process. Genes that lack a homolog are silenced during meiosis in *N. crassa* by MSUD (Shiu and Metzenberg, 2002). The *rec-2* and *rec-2*$^+$ sequences lack homology, so in a heterozygous mating neither has a homolog with which to pair (F. J. Bowring and D. E. A. Catcheside, unpublished data). If MSUD is disabled in a *rec-2*$^+$/*rec-2* heterozygote,

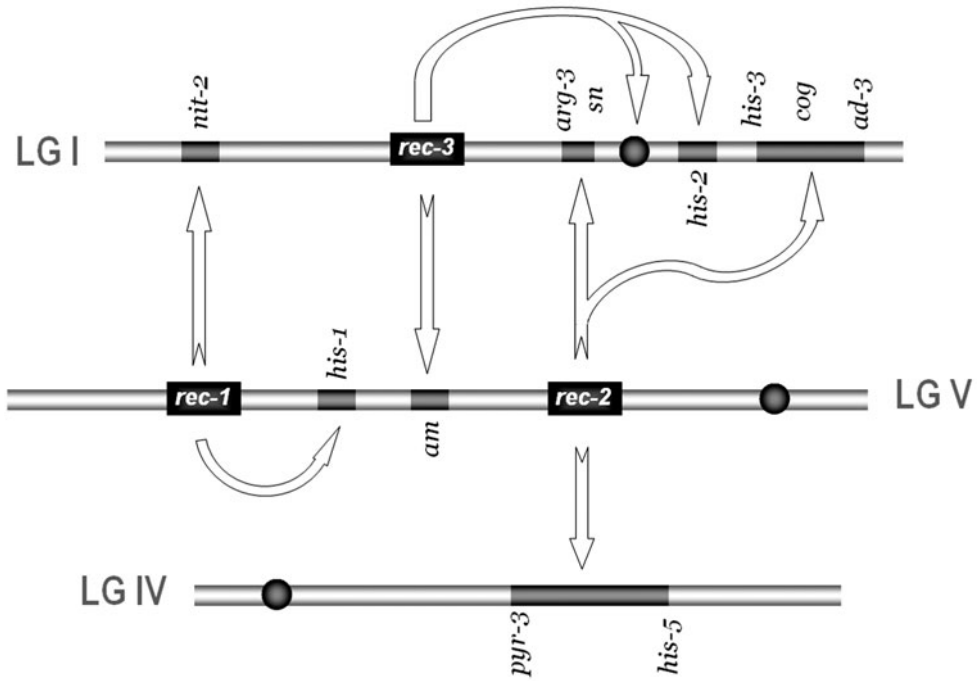

FIGURE 4 A partial linkage map of *Neurospora*, showing the locations of the known *rec* genes and the regions in which each *rec* gene influences recombination (arrow heads). Spheres represent the centromeres. The map is not to scale, and only part of each chromosome is shown.

recombination at *his-3* is substantially increased, although not quite to the level seen in a *rec-2* homozygote. Additionally, a *rec-2* deletion behaves, like *rec-2*$^+$, as a dominant suppressor of recombination.

Indeed, MSUD is responsible for the dominance of all three *rec* genes. In the absence of MSUD, recombination frequencies at *his-1* in *rec-1*$^+$/*rec-1* heterozygotes and at *am* in *rec-3*$^+$/*rec-3* heterozygotes are indistinguishable from those of *rec-1* and *rec-3* homozygotes, respectively (Bowring and Catcheside, unpublished). Thus, rather than *rec-n*$^+$ genes producing suppressors of recombination, the *rec-n* allele of each is an activator of recombination. Normal levels of recombination at the *rec-2* target near *his-3* have been observed in *spo11* mutants (see above), suggesting the tantalizing possibility that *rec* genes are a novel class of recombination initiators.

In addition to genes having a large effect on recombination, others having small effects are known in *N. crassa*. For example, both allelic recombination in *his-3* alleles and crossing-over in the vicinity varies at least twofold in crosses with the same *cog* and *rec-2* genotype (Yeadon et al., 2004a) depending on an unlinked factor in the genetic background. A similar effect is seen at the *nit-2* locus (Catcheside, 1970). It is not known if these genes of minor effect act locally or globally across the whole genome. It appears that *N. crassa* is highly polymorphic for genes with both large and small effects on recombination, suggesting there is selective advantage to the species in the ability to vary recombination frequency.

A and *B* Mating Type Factors in *Schizophyllum commune*

In *S. commune*, two unlinked factors, *A* and *B*, must both be heterozygous for a pair of strains to be compatible for mating (Simchen, 1967). The *A* factor is encoded at two linked loci, α and β (Raper et al., 1958, 1960), and recombination in the α to β interval is determined in the main by a single unlinked *rec* locus. In the presence of the dominant *rec*$^+$ allele, the frequency of recombination between α and β is reduced from an average of 15 to 4% (Simchen, 1967). There is also an effect of genetic background, as recombination frequencies vary from 1 to 8% in crosses including *rec*$^+$ and from 8 to 25% in the absence of *rec*$^+$.

There is a similar but independent system regulating recombination in the *B* factor. In addition, temperature has a differential effect on each regulatory system, suggesting that each *rec* gene product involved may have a different temperature optimum (Stamberg, 1968).

Sequence Heterology and Recombination

At the *b2* locus of *A. immersus*, sequence heterology has been used to infer details of recombination intermediates (Nicolas and Rossignol, 1989, and references therein). Recombination, and thus hDNA, is usually initiated at the end of the gene closer to the 1F1 mutation and extends towards A4 at the other end of the gene, but it can also be initiated elsewhere. Asymmetric hDNA is seen most often close to the initiator, declining towards the other end of the gene, while the gradient for symmetric hDNA is in the opposite direction.

Although extension of asymmetric hDNA is unaffected, heterology blocks extension of symmetric hDNA. Close to the initiator, mismatches in *b2* reduce both kinds of hDNA, perhaps by interfering with the strand invasion step that occurs immediately after initiation (Nicolas and Rossignol, 1989). Similarly, naturally occurring mismatches close to *cog* may interfere with strand invasion (Yeadon et al., 2004a), and mismatches within *his-3* appear to reduce the chance of resolution of a recombination intermediate between the mutant alleles (Yeadon et al., 2004a).

In *N. crassa*, heterozygosity for *TK* inserted between *cog* and *his-3* has no effect on allelic recombination in *his-3* (Yeadon et al., 2004a). These data suggest that recombination initiated at *cog*, after strand invasion and before resolution of the recombination intermediate, may involve only DNA synthesis and thus extension of asymmetric hDNA, also unaffected by heterology in *A. immersus* (Nicolas and Rossignol, 1989). In *A. immersus*, however, such large heterologies increase crossing-over, which tends to occur between the initiator and the heterology, suggesting it may block the migration of Holliday junctions and force their resolution (Nicolas and Rossignol, 1989). In contrast, heterozygosity for *TK* does not stimulate crossing-over in the *his-3* region of *N. crassa* but reduces the *his-3* to *ad-3* genetic interval by 50%. Some of the reduction in crossing-over is due to deletion of a novel crossover hot spot in the region replaced by *TK* (see below), but the heterology has an additional effect, which may not be confined to the heterologous region alone (Yeadon et al., unpublished).

A Novel Crossover Hot Spot near *his-3* in *Neurospora*

Using a *Neurospora* cross maximized for sequence heterology in the *his-3* region, high-density SNP analysis and partial sequencing of 150 octads were used to locate crossovers between *his-3* and *ad-3*. Crossovers appeared to have a nonrandom distribution, most falling close to *his-3* or more than 40 kb distal, and all those in which the location was determined flanked regions showing gene conversion and/or PMS. More crossovers than expected occurred within a 200-bp region that falls within the native sequence removed in the deletion/replacement strains described above (Yeadon et al., 2002).

The 200-bp sequence has been deleted in several strains to enable comparisons between crosses that are isogenic apart from the deletion. The deletion decreases crossing-over in the distal *his-3* to *ad-3* interval, increases recombination in the *his-3* to *lys-4* interval, and increases conversion in *his-3* more than would be expected (Yeadon et al., 2002) as a result of decreasing the distance from *cog* by 200 bp (Yeadon et al., unpublished). We conclude that the 200-bp sequence includes a motif that is a preferred site for termination of recombination events, so that when it is deleted, fewer events terminate between *his-3* and *cog*, lowering the crossover frequency in this interval. Conversely, more events reach *his-3* (so more conversion occurs), and more extend past *his-3*, thus increasing the frequency of crossovers in the proximal *lys-4* to *his-3* interval.

CONCLUSION

The availability of sequenced genomes for a number of filamentous fungi together with the development of efficient gene targeting techniques makes reverse genetics, previously very difficult with these organisms, relatively easy. Research into DNA repair and recombination can now proceed apace by deletion of each annotated gene with a predicted role in these processes, enabling us to move beyond understanding individual repair systems to an understanding of more complex networks. However, deletions alone will not be enough. As considered above, the location and type of a mutation can yield a novel phenotype. Moreover, epigenetic control of DNA metabolism will undoubtedly play a part in DNA repair and recombination.

Thus, the isolation of mutants sensitive to mutagens remains an indispensable tool if we are to develop a comprehensive understanding of DNA repair and recombination.

It is now clear that there are several pathways by which recombination occurs, that yeast does not contain them all, and that pathways present only in other species need to be investigated in detail. The filamentous fungi have greater genetic complexity than yeast and are more similar to complex organisms such as mammals. The filamentous fungi will continue to provide efficient model systems for such investigation.

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9

Chromatin Structure and Modification

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Chromatin is the substrate for virtually all DNA-mediated processes in eukaryotic cells. The basic unit of chromatin is the nucleosome, which is composed of ~146 bp of DNA wrapped around a protein core composed of histones H2A, H2B, H3, and H4 (Luger et al., 1997). The core histones are subjected to extensive posttranslational modification, which can influence interactions of nucleosomes with each other or with nonhistone chromatin proteins (Bhaumik et al., 2007; Jenuwein and Allis, 2001; Kouzarides, 2007). Replacement of the core histones with histone variants also contributes to the assembly of functionally distinct chromatin domains (Henikoff et al., 2004), while chromatin remodeling can further modulate chromatin structure (Cairns, 2007; Smith and Peterson, 2005). In addition, some organisms carry out methylation of cytosine bases in DNA to yield 5-methylcytosine (Klose and Bird, 2006). Ultimately, all of these processes contribute to the establishment of local chromatin environments that promote or inhibit various DNA-templated processes. In general, histones and chromatin modifiers are quite conserved, but there is some variation, even among the fungi. In this chapter, what is known about chromatin structure in the filamentous fungi is discussed. This information is compared to what is known for other eukaryotes (e.g., the extensively characterized budding yeast *Saccharomyces cerevisiae*), and exciting areas for future research are highlighted.

HISTONES

In budding yeast, transcription of the core histones is tightly regulated and is primarily restricted to the S phase of the cell cycle (Gunjan et al., 2005). Replication-dependent incorporation of newly synthesized histones occurs immediately behind the replication fork and involves the trimeric chromatin assembly factor 1 (CAF-1) (Krude and Keller, 2001). Components of CAF-1 are conserved in filamentous fungi and likely participate in replication-dependent chromatin assembly as well (Borkovich et al., 2004). CAF-1 can also serve as a scaffold to mediate interaction of proteins behind the replication fork. For example, the meiosis-specific recombinase Lim15/Dmc1 interacts with the large subunit of CAF-1 in *Coprinus cinereus* (Ishii et al., 2008). This interaction likely facilitates chromatin assembly following recombination. The genomes of many eukaryotes contain multiple copies of each histone gene. While transcription of some histone genes is restricted to S phase, others are transcribed throughout the cell cycle (Takayama and Takahashi, 2007). Notably, a distinct set of histone chaperones that occur in filamentous fungi catalyzes replication-independent histone deposition outside S phase (Borkovich et al., 2004; Henikoff and Ahmad, 2005; Tagami et al., 2004). Despite tight regulation of histone gene transcription, even budding yeast carries out replication-independent histone deposition at some regions of the genome (Green et al., 2005). Replacement of histones in this fashion serves to alter chromatin structure in one of two ways. Histones that have undergone posttranslational modification can be replaced by those that have not, or the canonical histone proteins can be replaced by histone variants.

The full complement of histone genes has been characterized for *Neurospora crassa* (Hays et al., 2002). *N. crassa* encodes a single gene for three of the canonical histones H3, H2A, and H2B, plus two copies of the canonical H4 protein, a single homolog of the centromere-specific H3 variant H3v/CENH3, and one H2A variant, H2Az. *N. crassa* also encodes a divergent H4 variant that is probably a pseudogene. Like other eukaryotes, the *hH3* and *hH4.1* genes are divergently transcribed from a shared promoter. *hH2A* and *hH2B* are also divergently transcribed. Independent inactivation of either *hH4.1* or *hH4.2*, which encode identical proteins, does not noticeably perturb growth. BLAST searches of the *Magnaporthe grisea*, *Aspergillus nidulans*, and *Fusarium graminearum* genomes suggest that the number and organization of histone genes are conserved in these fungi. In contrast, BLAST searches of the basidiomycete *C. cinereus* genome suggest that it has multiple copies of both *hH3-hH4* and *hH2A-hH2B* gene pairs. Table 1 details the complement of predicted histone genes found in the genome sequences of *N. crassa* and *C. cinereus* (http://www.broad.mit.edu/science/data#).

The functions of the H3 and the H2A variants are not currently established for filamentous fungi. Like its homologs in yeast, plants, and animals, the H3 variant is

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TABLE 1 Histone genes in *N. crassa* and *C. cinereus*

Histone	*N. crassa* gene[a]	*C. cinereus* gene[b]
H3	NCU01635.3	CC1G_05766.2
		CC1G_05755.2
		CC1G_08799.2
		CC1G_04396.2
H4	NCU01634.3	CC1G_05765.2
	NCU00212.3	CC1G_05756.2
		CC1G_05458.2
H2A	NCU02437.3	CC1G_07640.2
		CC1G_03522.2
		CC1G_03575.2
H2B	NCU02435.3	CC1G_07639.2
		CC1G_03523.2
H1	NCU06863.3	CC1G_03813.2
		CC1G_03810.2
		CC1G_13972.2
H3v/CENH3	NCU00145.3	CC1G_00384.2
H2Az	NCU05347.3	CC1G_08736.2

[a]Data from Hays et al., 2002.

[b]These sequences were identified by performing BLAST searches of the *C. cinereus* genome sequence, using the corresponding *N. crassa* histone protein sequences as the query sequence.

presumably involved in centromere function, serving as the site of kinetochore assembly (Black and Bassett, 2008). The function of the H2A variant H2Az is not well understood in any organism. In yeast, this protein localizes to promoter regions and acts to prevent spreading of heterochromatin (Eirin-Lopez and Ausio, 2007). Similarly, H2Az appears to antagonize DNA methylation in plants (Zilberman et al., 2008). In *Drosophila* and mammals, H2Az also localizes to heterochromatic regions and may be important for heterochromatin formation or function (Zlatanova and Thakar, 2008). It is possible that H2Az has evolved different functions in different eukaryotic lineages. Investigation of H2Az function in filamentous fungi is an exciting area for future research.

Higher eukaryotes encode two noncentromeric histone H3 proteins (Henikoff and Ahmad, 2005). Canonical H3 is expressed during S phase and incorporated into chromatin in a replication-dependent manner. In contrast, the histone variant H3.3 is transcribed outside S phase and is incorporated into histones in a replication-independent manner. Fungi encode a single canonical H3, which belongs to the H3.3 group based on amino acid sequence. As mentioned above, however, work with budding yeast revealed that distinct groups of histone chaperones can catalyze deposition of this H3 protein in replication-dependent or -independent fashions (Green et al., 2005), and both sets of chaperones are present in the genome sequences of filamentous fungi (Borkovich et al., 2004). Thus, nonreplicative histone replacement may serve to erase preexisting chromatin marks in filamentous fungi as well.

The linker histone H1 is currently the most extensively characterized histone in filamentous fungi, but much is still unknown about the function of this protein. Study of plant and animal H1 has been primarily carried out in vitro because higher eukaryotes encode multiple linker histones, which complicates in vivo depletion studies. Indeed, the human genome encodes 11 H1 variants (Izzo et al., 2008). The genomes of *S. cerevisiae* and *Tetrahymena thermophila* possess single H1 genes, but the encoded proteins have rather different structures from those in higher eukaryotes. Thus, it is not clear if these proteins are functionally equivalent to their counterparts in higher eukaryotes. The fission yeast *Schizosaccharomyces pombe* lacks an obvious homolog of H1. Like budding yeast, several ascomycete genomes contain single *hH1* genes, yet the H1 proteins encoded by these fungi are structurally more similar to those of higher eukaryotes (Barra et al., 2000; Brosch et al., 2008; Folco et al., 2003; Ramon et al., 2000). BLAST searches of the *C. cinereus* genome reveal three putative H1 proteins, suggesting that basidiomycetes may be more complex than their ascomycete counterparts. *A. nidulans*, *N. crassa*, and *Ascobolus immersus* strains harboring inactive copies of *hH1* are viable, indicating that H1 does not perform essential functions in these fungi. Deletion of the *hho*A gene from *A. nidulans* produced no observable phenotypes (Ramon et al., 2000). An *A. immersus* strain harboring a MIP-silenced copy of *H1* produced three observable phenotypes, an overall increase in nuclease accessibility, DNA hypermethylation, and a decrease in lifespan (Barra et al., 2000) (see "Silent Chromatin and DNA Methylation" below for an explanation of MIP). In *N. crassa*, mutants lacking H1 exhibited transcriptional derepression of the gene encoding pyruvate decarboxylase (*cfp*), and a transcriptional fusion of the *cfp* promoter to the *hygromycin phosphotransferase* (*hph*) gene produced elevated *hph* expression in the *hH1* mutant (Folco et al., 2003). No effects on DNA methylation were observed. It is possible that H1 acts as a sequence-specific transcriptional repressor in fungi, but more-detailed analysis of H1 is required to determine the specific function of this histone.

HISTONE MODIFICATIONS

Posttranslational modification of histone proteins by acetylation and methylation was first described in 1964 (Allfrey et al., 1964). Acetylated histones supported higher rates of RNA synthesis than did unacetylated histones, suggesting that posttranslational modification of the histone proteins could produce functionally distinct chromatin domains. Indeed, the identification of histone modifications and their functions has been an intense and fruitful area of investigation. The expanding list of modifications now includes acetylation, methylation, phosphorylation, ADP-ribosylation, deimination (arginine to citrilline), proline isomerization, ubiquitylation, and sumoylation (Bhaumik et al., 2007; Jenuwein and Allis, 2001; Kouzarides, 2007; Latham and Dent, 2007). Some representative modifications that occur within the N-terminal tail domains of H3 and H4 are shown in Fig. 1. Many histone "marks" are reversible, a feature that contributes to the dynamic nature of the chromatin fiber. Further complexity results from the fact that lysine residues can be mono-, di-, or trimethylated, and each state may have distinct functional consequences. Similarly, monomethylated, symmetrically dimethylated, and asymmetrically dimethylated forms of arginine have been identified. Genetic and biochemical studies have led to the identification of many of the enzymes that establish these modifications, as well as those that reverse them.

Modification of histones may impact nuclear processes through at least two mechanisms. (i) The modifications themselves can influence interactions between nucleosomes, thus promoting or inhibiting the assembly of higher-order chromatin structures. (ii) Alternatively, the modifications can create binding sites for protein complexes that directly

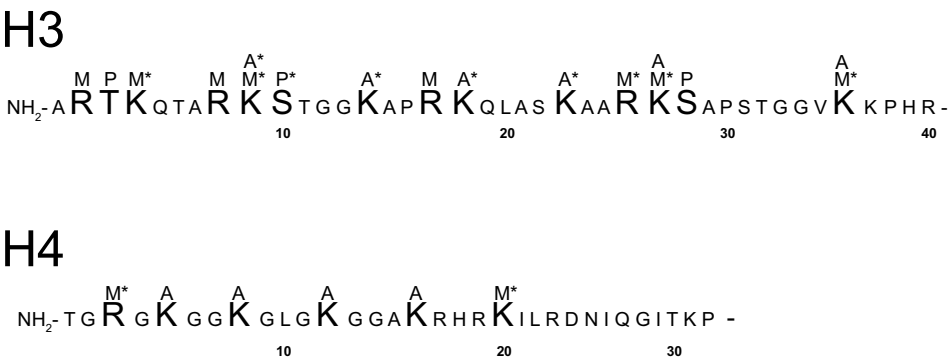

FIGURE 1 Covalent modifications of the H3 and H4 N-terminal tails. The amino acid sequence of the N-terminal tails of H3 and H4 is shown. Some of the modified residues are shown in larger font, and modification of these residues by acetylation (A), methylation (M), or phosphorylation (P) is indicated above the respective amino acids. The modifications highlighted by an asterisk have been characterized in filamentous fungi.

catalyze DNA-templated reactions or complexes that further modify chromatin structure. Indeed, several protein domains that recognize and bind specific histone modifications have been identified, supporting the hypothesis that a "histone code" consisting of chromatin marks and proteins that read them serves to link chromatin structure and function (Jenuwein and Allis, 2001; Taverna et al., 2007).

Histone modifications can be loosely grouped into "active marks," which facilitate processes such as transcription, recombination, and DNA repair, and "repressive marks," which tend to inhibit these processes. Identification of modified residues and their functions has progressed more rapidly for H3 and H4, in part because H3 and H4 are more conserved than H2A and H2B. Indeed, antibodies that specifically recognize modifications of H3 or H4 can be used experimentally in a wide range of eukaryotic species. Furthermore, studies have shown that the functions of several histone modifications are conserved across eukaryotic kingdoms (Kouzarides, 2007). Modifications of H2A and H2B have also been identified, and some of these modifications appear to act in conserved pathways (e.g., ubiquitylation of H2B plays a conserved role in transcription in yeast, plants, and humans) (Weake and Workman, 2008). In general, transcriptionally active chromatin contains hyperacetylated histones and H3 methylated at K4, K79, and K36. Conversely, silent chromatin contains hypoacetylated histones and H3 methylated at K9 (Bhaumik et al., 2007; Kouzarides, 2007).

Fungi are excellent models to elucidate the function of histone modifications. Unlike higher eukaryotes, the genomes of many fungi have, or are predicted to have, single enzymes that catalyze a specific histone modification. For example, whereas methylation of H3 K9 is catalyzed by at least four separate enzymes in mammals (Lall, 2007), a single protein appears to be responsible for all detectable H3 K9 methylation in *N. crassa* (Tamaru and Selker, 2001; Tamaru et al., 2003). Despite this advantage, our understanding of chromatin modifications and their functions in filamentous fungi is in its infancy.

In eukaryotes, virtually all histone lysine methylation is performed by enzymes that contain a SET domain (Bhaumik et al., 2007). The only known exception is methylation of H3 K79, which is catalyzed by a non-SET domain protein, DOT1. DOT1 homologs are present in filamentous fungi, as are a number of SET domain proteins (Adhvaryu et al., 2005; Borkovich et al., 2004; Brosch et al., 2008; Palmer et al., 2008; Tamaru and Selker, 2001). Analysis of H3 and H4 from *N. crassa* revealed that H3 is methylated at lysines 4, 9, 27, 36, and 79, whereas H4 is methylated at lysine 20 (Adhvaryu et al., 2005; Tamaru and Selker, 2001). It is unlikely that this list is complete, since detection of these modifications relied on commercially available antibodies. In filamentous fungi, H3 K9 methylation, which is a component of silent heterochromatin, represents the most extensively characterized histone modification (discussed below). Initial examination of H3 K4 and K36 methylation indicates that these modifications have conserved functions in transcription (Adhvaryu et al., 2005).

CHROMATIN AND TRANSCRIPTION

In yeast, animals, and plants, H3 K4 methylation is associated with expressed genes (Bhaumik et al., 2007; Kouzarides, 2007). Chromatin immunoprecipitation experiments in *N. crassa* indicated that H3 dimethyl K4 associates with at least three expressed genes, namely, *mtr*, *hH4*, and *pcna* (Adhvaryu et al., 2005; Honda and Selker 2008; Tamaru et al., 2003). Similarly, in *A. immersus* H3 dimethyl K4 is found in expressed genes but is absent from these genes when they are silenced by DNA methylation (Barra et al., 2005). Chromatin immunoprecipitation followed by hybridization to a high-resolution microarray covering *N. crassa* LGVII confirmed that H3 dimethyl K4 resides within genes (Lewis et al., 2009). Indeed, nearly 45% of LGVII genes were enriched for H3 dimethyl K4, while this modification appeared to be excluded from heterochromatin domains (Fig. 2).

In *S. cerevisiae*, methylation of H3 K36 is enriched within the 3′ regions of transcribed genes, where it recruits the histone deacetylase (HDAC) Rpd3 (Carrozza et al., 2005; Keogh et al., 2005). Why would a mark associated with active transcription recruit a protein associated with transcriptional repression? It turns out that deacetylation by Rpd3 prevents RNA polymerase II transcript initiation at cryptic promoters that reside within coding sequences. H3 K36 methylation may serve a similar function in filamentous fungi. Adhvaryu and colleagues inactivated the gene encoding SET2 in *N. crassa* and demonstrated that this enzyme catalyzes methylation of H3 K36 (Adhvaryu et al., 2005). *set-2* mutants exhibited defects in sexual and asexual

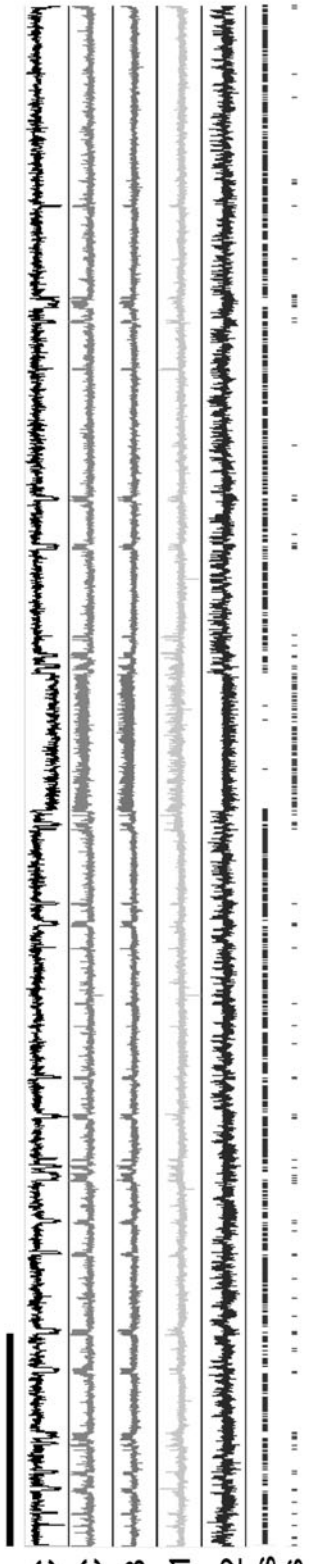

FIGURE 2 Chromatin modification profile for *N. crassa* LGVII. The base composition of LGVII is shown as the moving average of %GC at the top of the plot. The distribution of DNA methylation (5mC), H3 trimethyl K9 (H3K9me3), HETEROCHROMATIN PROTEIN-1 (HP1), and H3 dimethyl K4 (H3K4me2) is shown for LGVII. The positions of predicted open reading frames (genes) and repeats are also indicated. The scale bar on the top left indicates 0.5 Mb.

development, suggesting that SET-2 activity is important for expression of genes involved in these developmental pathways. Introducing a mutant histone harboring a K36-to-L substitution reproduced the set-2 phenotype. Notably, chromatin immunoprecipitation assays demonstrated that K36 methylation was enriched in the 3′ region of the *mtr* gene, suggesting that K36 methylation may serve similar functions in budding yeast and filamentous fungi.

Changes in the position of individual nucleosomes can expose or mask regulatory sites within a DNA molecule (Cairns, 2007). This process, called chromatin remodeling, is often observed within eukaryotic promoters and can even include complete ejection of nucleosomes from the chromatin fiber. Such chromatin remodeling is frequently correlated with changes in transcription. Indeed, many promoter-associated chromatin domains adopt an "open" conformation following activation of transcription. Changes in chromatin structure from a closed to an open conformation may result from transcription. Alternatively, remodeling of promoter nucleosomes may represent a distinct, regulated event that occurs prior to transcription. Several elegant studies have addressed these possibilities by examining nucleosome remodeling at inducible promoters in species of *Aspergillus*.

In *A. nidulans*, expression of the divergently transcribed *niaD* and *niaA* genes requires induction by nitrate (mediated by NirA) and derepression in the absence of ammonium (mediated by AreA). Within the *niaD-niaA* promoter, a nucleosome-free region containing binding sites for AreA is flanked on either side by positioned nucleosomes (Muro-Pastor et al., 1999). Activation of transcription results only when induction by nitrate is accompanied by loss of repression (i.e., the absence of ammonium). Induction of transcription results in remodeling of the entire nucleosome array in a manner that depends on AreA. Importantly, these changes occur in the absence of the activator NirA, indicating that transcription is not required to induce chromatin remodeling.

Similarly, disruption of an ordered nucleosome array at the *alcA* and *alcR* promoters occurs following transcriptional induction and requires the transcriptional activator AlcR (Mathieu et al., 2000). Since deletion of the *alcA* TATA box produced a dramatic reduction in the rate of transcription but did not inhibit the AlcR-dependent changes in nucleosome position, the authors concluded that chromatin remodeling and transcriptional activation occur in a stepwise fashion at the *alcA* promoter.

Several studies have explored the relationship between histone acetylation and chromatin remodeling. For example, analysis of the bidirectional *prnD-prnB* promoter from *A. nidulans* revealed that the ordered pattern of nucleosomes observed under repressed conditions was perturbed by treatment with the HDAC inhibitor trichostatin A (TSA) (Garcia et al., 2004). Moreover, induction of *prnD-prnB* transcription is accompanied by increases in acetylation of H3 K9 and K14 in addition to rearrangement of nucleosomes at the promoter (Reyes-Dominguez et al., 2008). Chromatin remodeling at the promoter does not require acetylation of these residues, however, since mutant strains lacking the GcnE or AdaB components of the SAGA histone acetyltransferase (HAT) complex display normal induction of transcription and normal nucleosome remodeling at the promoter. The authors speculate that perhaps acetylation of other residues in H3 or acetylation of H4, H2A, or H2B by additional HATs could be required for chromatin remodeling upon transcriptional activation.

Similarly, AreA-dependent acetylation of H3 K9 and K14 is associated with chromatin remodeling at the *niaD-niaA* promoter (Berger et al., 2008). Under certain nutrient conditions, however, NirA can induce chromatin remodeling that is not dependent on increased acetylation of H3 K9 and K14. This suggests that acetylation is not required for all chromatin remodeling at this promoter or alternatively, acetylation of other lysine residues on H3, H4, H2A, or H2B may be important for NirA-mediated chromatin remodeling at *niaD-niaA*.

Although involvement of SAGA was not required for remodeling at the *A. nidulans prnD-prnB* promoter, studies of the light-induced *albino-3* gene in *N. crassa* uncovered an essential role for histone acetylation at this promoter (Grimaldi et al., 2006). Light-induced transcription at the *N. crassa al-3* promoter was accompanied by an increase in acetylation of H3 K14, dependent on the WC-1 transcriptional activator. Moreover, *N. crassa* strains expressing a mutant H3 protein with a K14-to-Q substitution display reduced gene activation in response to light, suggesting that acetylation of this residue is required for normal transcription induction. Lastly, light-induced acetylation at the *al-3* promoter and light-induced transcription of the *al-3* and *vivid* genes were abolished in a mutant strain lacking the *N. crassa* Gcn5 homolog NGF-1.

Nucleosome position is determined by DNA sequence, the occupancy of DNA binding proteins, and the activity of chromatin remodeling enzymes (Schnitzler, 2008). Indeed, the *A. nidulans* CCAAT binding factor AnCF and its binding site are both required to organize a nucleosome-free region within the *amdS* promoter (Narendja et al., 1999). Similarly, two DNA sequence motifs within the *Hypocrea jecorina cbh2* promoter are required to properly position nucleosomes at this promoter (Narendja et al., 1999).

The chromatin remodelers that catalyze the dramatic changes in nucleosome organization at the *Aspergillus* promoters described above have yet to be identified. In *N. crassa*, however, knockout mutants for 19 predicted ATP-dependent chromatin-remodeling enzymes have been created (Belden et al., 2007). One of these enzymes, named CLOCKSWITCH (CSW-1), is required for normal functioning of the circadian clock. Binding of CSW-1 to the promoter of the *frequency* (*frq*) gene occurs in a circadian fashion. Light-induced and circadian-cycle-regulated expressions of *frq* are respectively mediated by two *cis*-acting elements within the promoter, the light response element and the clock box (C-box) (Froehlich et al., 2002). Light induction produces a change in micrococcal nuclease accessibility near the light response element (Belden et al., 2007). Likewise, a change in micrococcal nuclease accessibility is observed near the C-box over the course of a circadian cycle. These circadian changes in the *frq* promoter were reduced, but not abolished, in the *csw-1* mutant strain. The authors conclude that CSW-1 acts to negatively regulate binding of the transcription factor WC-2 to the C-box by remodeling chromatin at this promoter element. Interestingly, circadian and light-regulated chromatin rearrangements at the *frq* promoter persist in the *csw-1* mutant strain, indicating that multiple chromatin-remodeling enzymes act on the *frq* promoter.

Histone acetylation is typically associated with transcriptional activation, while removal of acetyl groups by HDACs is frequently involved in transcriptional repression. Eukaryotic genomes encode multiple HDACs that fall into four categories (Brosch et al., 2008; de Ruijter et al., 2003). Class I and II HDACs are homologs of *S. cerevisiae* RPD3 and HDA1, respectively. Homologs of the *S. cerevisiae* NAD^+-dependent HDAC SIR2, called sirtuins, constitute the class III HDACs. Lastly, class IV HDACs, which are absent from fungi, include HD2 of plants and HDAC11 in mammals.

A. nidulans RpdA and HosA were the first HDACs identified in filamentous fungi (Graessle et al., 2000), followed shortly by identification of their respective homologs HDC2 (Brosch et al., 2001) and HDC1 (Baidyaroy et al., 2001) in *Cochliobolus carbonum*. Mutants lacking HDC1 displayed reduced virulence, abnormal conidium production, reduced growth rates on carbon sources other than glucose, and a ~50% reduction in global HDAC activity (Baidyaroy et al., 2001). Surprisingly, the reduced growth rates were correlated with a decrease in expression of several extracellular depolymerases. Loss of expression did not result from derepression of the carbon catabolite repressor CREA. Why HDC1, a presumed transcriptional repressor, is required for transcription activation is not clear, but this represents an interesting question to address in future studies. Similarly, studies with *Ustilago maydis* also revealed a role for the RPD3 homolog Hda1 in both repression and activation of genes, as well as an essential role for this protein in teliospore development (Reichmann et al., 2002; Torreblanca et al., 2003).

S. cerevisiae HOS3 has sequence features attributed to both Class I and Class II enzymes and therefore cannot readily be grouped into either of these classes (Brosch et al., 2008). While HOS3 homologs have not been identified in plant or animal systems, HOS3 homologs HosB and HDC4 were identified in *A. nidulans* and *C. carbonum*, respectively (Trojer et al., 2003). Homologs are also present in the genome sequences of other ascomycetes and several basidiomycetes, suggesting that this group of HDACs is specific to fungi (Brosch et al., 2008). Like HOS3 in budding yeast (Carmen et al., 1999), the HDAC activity of *A. nidulans* HosB was highly resistant to HDAC inhibitors TSA and HC-toxin (Trojer et al., 2003).

Interestingly, the HDAC inhibitor HC-toxin is a secondary metabolite (SM) produced by *C. carbonum*. Other filamentous fungi such as *Alternaria brassicicola* also produce SM HDAC inhibitors. It is possible that HOS3 family HDACs are upregulated in response to exposure to these inhibitors. This is not the only mechanism of self-protection, however. *C. carbonum* apparently produces an extrinsic factor that serves to protect its normally sensitive HDACs from HDAC inhibitors (Baidyaroy et al., 2002).

Trojer and colleagues identified the class II HDACs HdaA from *A. nidulans* and HDC3 from *C. carbonum*. Analyses of chromatography fractions containing HDAC activity from wild-type and *hdaA* mutant strains indicated that HdaA accounts for the majority of HDAC activity in *A. nidulans*, while RpdA accounts for most of the remaining activity (Tribus et al., 2005; Trojer et al., 2003). In addition, these analyses demonstrated that HosA and an NAD^+-dependent activity, likely due to a SIR2 homolog, also contribute to the overall HDAC activity under standard laboratory growth conditions (Trojer et al., 2003). The contributions of these enzymes are minor relative to those of HdaA and RpdA. The authors also demonstrate that HdaA, HosA, and RpdA are all present in high-molecular-mass complexes. Strains that lack HdaA are sensitive to oxidative stress, which correlated with decreased transcription of the catalase *catB* gene and reduced activity of the CatB enzyme (Tribus et al., 2005). These effects on *catB* transcription are presumably indirect but are not currently understood at the molecular level.

Notably, more recent work revealed that HdaA is involved in repression of telomere-proximal SM clusters in *A. nidulans* (Shwab et al., 2007). Examination of SM gene expression in mutants lacking *A. nidulans* HosB or the SIR2 homolog HstA revealed no effects, but a triple mutant lacking HdaA, HosB, and HstA showed enhanced SM gene transcription relative to the *hdaA* mutant alone. These data suggest that the deacetylases work in concert to regulate genes from at least two telomere-linked SM clusters. Furthermore, chromatin immunoprecipitation experiments with *Aspergillus parasiticus* revealed that ordered expression of genes within the aflatoxin gene cluster is accompanied by acetylation of H4 (Roze et al., 2007). Taken together, these data suggest that an antagonistic relationship between HATs and HDACs is important for the coordinated regulation of SM genes in filamentous fungi.

Histone arginine methylation has been shown to positively and negatively regulate transcription in eukaryotes (Bedford 2007; Bedford and Richard, 2005). The genomes of *A. nidulans* and *N. crassa* each encode three predicted protein arginine methyltransferases (PRMT) (Borkovich et al., 2004; Trojer et al., 2004). The *A. nidulans* and *N. crassa* proteins RmtA/PRMT1, RmtB/PRMT3, and RmtC/PP-2 most closely resemble higher-eukaryotic PRMT1, PRMT3, and PRMT5, respectively. Notably, RmtB and PRMT3 lack an N-terminal Zn-finger domain that is present in PRMT3 homologs in higher eukaryotes. Functional analysis of the *A. nidulans* PRMTs revealed that all three proteins methylate histone substrates in vitro (Trojer et al., 2004). RmtA specifically methylates H4, RmtB methylates H4, H3, and H2A, and RmtC methylates H4 and H2A. The authors further demonstrated that all three PRMT proteins methylate H4 R3 and RmtB methylates H3 R26. The list of substrate residues is likely incomplete since antibodies were available only for methylated H4 R3, H3 R17, and H3 R26. Examination of histones isolated from *A. nidulans* revealed that methylation of H4 R3 and H3 R26 occurs in vivo but H3 R17 methylation does not. Moreover, in vitro methylation of H4 R3 by RmtA is inhibited when histones are first acetylated by the p300 HAT. Similarly, methylation of H3 by RmtB inhibits subsequent acetylation of histones by recombinant p300. In contrast, methylation of H4 by RmtB enhances acetylation by p300. Also, acetylation of H3 and H4 does not inhibit the methyltransferase activity of RmtB. Future studies that examine the function of these PRMT proteins in vivo will advance our understanding of the role of histone arginine methylation in filamentous fungi and in other eukaryotes.

SILENT CHROMATIN AND DNA METHYLATION

Heterochromatin is typically rich in repeated sequences and often comprises important structural domains, such as centromeres and telomeres. With respect to heterochromatin, the filamentous fungi are more similar to higher eukaryotes than to the budding yeast. Heterochromatin formation and maintenance in *S. cerevisiae* relies on a complex of Sir proteins (Rusche et al., 2003), only one of which, the NAD^+-dependent HDAC Sir2, is conserved in higher eukaryotes (Dali-Youcef et al., 2007). In contrast, H3 methylated at K9 is a mark for heterochromatin in the fission yeast *S. pombe*, plants, and animals but is absent from budding yeast (Grewal and Jia, 2007). Methylation of H3 K9 has been documented in several filamentous fungi (Barra et al., 2005; Palmer et al., 2008; Tamaru and Selker, 2001). In addition, the protein that reads this mark, HETEROCHROMATIN PROTEIN-1, is also absent from budding yeast but is present in the genomes of higher eukaryotes and filamentous fungi (Brosch et al., 2008; Freitag et al., 2004). Similarly, many genetically tractable model organisms such as budding yeast, fission yeast, *Drosophila*, and *Caenorhabditis elegans* lack readily detectable DNA methylation, which is a common feature of heterochromatin and repeated sequences in plants, animals, and some filamentous fungi (Suzuki and Bird, 2008). Thus, filamentous fungi are particularly attractive model systems to investigate heterochromatin formation and function due to their similarity to higher eukaryotes and their genetic tractability.

Much of our understanding of heterochromatin in filamentous fungi has emerged from the study of DNA methylation. The first methylated sequences identified in a fungal genome were a pair of tandemly arranged 5S rRNA pseudogenes in *N. crassa* (Selker and Stevens, 1985). These 5S rRNA pseudogenes are highly divergent and are rich in AT base pairs relative to other *N. crassa* 5S rRNA sequences. The discovery and subsequent characterization of the premeiotic genome defense system repeat-induced point mutation (RIP) revealed that these duplicated sequences were, in fact, relics of this process (Selker and Stevens, 1985). RIP operates exclusively during the sexual phase of the life cycle. After fertilization but prior to nuclear fusion, duplicated sequences within each haploid genome are detected and both copies are littered with C-G to T-A transition mutations. In the vegetative phase of the life cycle, products of RIP exhibit dense nonsymmetrical DNA methylation of most remaining cytosines (Selker et al., 1993a). In contrast, virtually all DNA methylation in animals occurs at symmetrical CpG dinucleotides and much of the methylation in plants occurs at symmetrical CpG or CpHpG (H=C, A, T) sites (Suzuki and Bird, 2008).

Similar to the situation in *N. crassa*, DNA methylation is targeted to sequence duplications in the ascomycete *A. immersus* by a RIP-like process termed methylation induced premeiotically (MIP) (Colot et al., 1996; Rossignol and Faugeron, 1995; Selker, 1997). In *A. immersus*, duplicated sequences detected during the premeiotic phase of sexual reproduction are heavily methylated, yet the primary DNA sequences remain unaltered. Thus, the DNA methylation established by MIP silences expression of duplicated sequences, but unlike what is observed with RIP, mutation of duplicated sequences does not occur.

The catalytic domains of both prokaryotic and eukaryotic cytosine DNA methyltransferases (DNMT) are composed of 10 highly conserved amino acid sequence motifs (Goll and Bestor, 2005), which led to the identification of two putative DNA methyltransferases from *A. immersus*. Masc1, the first DNMT homolog discovered in fungi, is the founding member of a family of fungus-specific DNMTs (Malagnac et al., 1997). Interestingly, Masc1 is required for MIP and for normal sexual development but is not required for maintenance of preexisting DNA methylation. These data suggest that Masc1 acts as a de novo DNMT during sexual development, while at least one maintenance methyltransferase acts to propagate preexisting methylation patterns during vegetative growth. Identification of the proposed maintenance methyltransferase(s) in *A. immersus* has proven difficult, however. Masc2, a second putative DNMT from *A. immersus*, exhibits C-5 DNMT activity in vitro, but mutant strains that lack this protein exhibit normal DNA methylation during vegetative and sexual phases of the life cycle (Malagnac et al., 1999). Additional work is necessary

to elucidate the proteins and mechanisms that contribute to the overall pattern of DNA methylation in this fungus.

In *N. crassa*, all DNA methylation within vegetative tissue requires the DNMT DIM-2 (Kouzminova and Selker, 2001). The DIM-2 protein is similar to *A. immersus* Masc2, and these proteins belong to a family of eukaryotic DNMTs that includes MET-1 from *Arabidopsis thaliana* and DNMT1 from mammals (Colot and Rossignol, 1999; Goll and Bestor, 2005). The finding that DIM-2 is responsible for all vegetative DNA methylation in *N. crassa* is notable for two reasons. First, this suggests that DIM-2 is capable of performing cytosine methylation at both symmetric and asymmetric sites. This is a unique feature of DIM-2, since animal and most plant methyltransferases exhibit strict substrate specificity (Goll and Bestor, 2005). Second, this observation revealed that DNA methylation is not essential in *N. crassa* (Kouzminova and Selker, 2001). In fact, the *dim-2* mutant displays apparently wild-type growth under all conditions that have been examined. This stands in contrast to the situation in plants and animals, where DNA methylation is essential for normal development.

One proposed mechanism of RIP involves cytosine methylation followed by deamination to produce C-to-T transition mutations (Galagan and Selker, 2004). Initial analysis of strains that lack DIM-2, however, revealed that RIP occurs normally in these strains. Searches of the *N. crassa* genome sequence revealed a second DNMT, which resembles *A. immersus* Masc1. Genetic analysis demonstrated that this DNMT homolog is required for RIP (Freitag et al., 2002). Hence, the protein was named RIP DEFECTIVE (RID). In contrast to *A. immersus* Masc1, RID is not required for sexual development, indicating that this is not a general function of RID/Masc1 family DNMTs.

Experimental analysis of DNA methylation and RIP in organisms other than *N. crassa* and *A. immersus* is in its early stages. The current data suggest that the filamentous fungi are a heterogeneous group with respect to both DNA methylation and RIP. DNA methylation of transposable elements or multiple-copy transgenes has been experimentally verified in a handful of fungi, including *M. grisea* (Nakayashiki et al., 2001), *Schizophyllum commune* (Mooibroek et al., 1990), and *C. cinereus* (Freedman and Pukkila, 1993). Searches of the genomic and expressed sequence tags (EST) data available for many filamentous fungi reveal that homologs of RID/Masc1 are widespread within the filamentous ascomycetes, but obvious homologs are not present in the genomes of the basidiomycete *C. cinereus* or of the yeasts *S. pombe* and *S. cerevisiae*. Although DNA methylation has not been detected in *A. nidulans*, its genome contains a single DNMT homolog that belongs to the RID/Masc1 family. Analysis of this gene, which was named *DNA methyltransferase homolog A* (*dmtA*), revealed an essential role in sexual development, similar to the case for Masc1 (Lee et al., 2008). The essential function(s) that DmtA and Masc1 perform is unknown. While DNA methylation has not been detected within *A. nidulans*, it is possible that trace amounts of transient DNA methylation are restricted to a few nuclei within the reproductive tissue. Alternatively, these proteins may have a function that does not require DNMT activity.

DNMTs of the DIM-2 family are found in several phylogenetically diverse species, including several ascomycetes such as *N. crassa*, *M. grisea*, and *F. graminearum*, as well the basidiomycete *C. cinereus*. In order to generate a more complete understanding of DNA methylation in fungi, experimental evidence of in vivo DNMT activity must be demonstrated for these predicted enzymes. It will also be important to better characterize the distribution of methylated cytosine within fungal genomes.

Currently, the genomic distribution of DNA methylation is best defined in *N. crassa*. Genomic analyses of DNA methylation clearly demonstrated an intimate association between RIP and DNA methylation. The first genome scale study to determine which sequences are methylated involved purification of methylated DNA by methylated DNA affinity chromatography (Selker et al., 2003). This study identified methylated sequences from all seven *N. crassa* chromosomes. Notably, virtually all of these sequences displayed hallmarks of RIP. More recently, analysis of the distribution of DNA methylation was performed by methylated DNA immunoprecipitation followed by hybridization to high-resolution DNA microarrays covering LGVII at 100-bp resolution (Lewis et al., 2009). DNA methylation on this chromosome was highly correlated with the presence of AT-rich DNA that had undergone the RIP process (hereafter referred to as "RIP'd") (Fig. 2). Notably, dense DNA methylation was detected within a large (~350-kb) centromeric domain, as well as in over 40 additional domains ranging from 0.5 to 20 kb. These included short domains adjacent to each telomere. All methylated domains exhibit hallmarks of RIP, reinforcing the earlier, less detailed study. No significant methylation was detected within genes, suggesting that this fungus does not rely on DNA methylation to regulate expression of functional coding sequences. Studies designed to address the function of DNA methylation in *N. crassa* and *A. immersus* showed that DNA methylation inhibits transcription elongation, but they did not reveal a role for DNA methylation at promoters. DNA methylation within the *mtr* and *am* promoters of *N. crassa* and the promoters of an *hph* reporter and the *met-2* gene in *A. immersus* did not inhibit transcription initiation, in contrast to the situation in mammals (Barra et al., 2005; Rountree and Selker, 1997). DNA methylation within the coding regions of these genes inhibited transcription elongation in both of these fungi, however.

Association of DNA methylation with sequences targeted by RIP and MIP is consistent with a mechanism that involves establishment of DNA methylation during the sexual phase of the life cycle, followed by maintenance methylation during vegetative growth. At least in *N. crassa*, however, it is clear that RIP'd DNA is a *cis*-acting signal for DNA methylation (Miao et al., 2000; Selker et al., 1993b; Selker and Stevens, 1987; Singer et al., 1995). Indeed, analysis of a series of RIP'd alleles of the *am* gene revealed that *N. crassa* performs both maintenance and de novo methylation (Selker et al., 2002; Singer et al., 1995). Whereas no methylation was detected in lightly RIP'd alleles of *am* during vegetative growth, moderately RIP'd sequences were methylated following RIP but did not support de novo methylation when unmethylated copies were reintroduced by transformation. In contrast, heavily RIP'd sequences were methylated following RIP and triggered de novo methylation when unmethylated copies were reintroduced by transformation. Dissection of sequences that are sufficient to target DNA methylation suggested that cooperative recognition of AT-rich sequences, potentially by an AT-hook domain protein, directs DNA methylation (Miao et al., 2000; Selker et al., 1993b; Tamaru and Selker, 2003).

Although the protein(s) that recognizes RIP'd DNA has not been identified, studies with *N. crassa* have uncovered a direct link between histone modification and DNA methylation. The SET domain protein DIM-5 is required for all detectable DNA methylation in this fungus (Tamaru

and Selker, 2001). DIM-5 most closely resembles *S. pombe* Clr4 and animal SUVAR 3–9 proteins but is unique in that it lacks the N-terminal chromo domain that is present in these proteins. In contrast to *dim-2* strains, which display no defects in growth or development, *dim-5* mutants display reduced growth rates and exhibit defects in sexual development. The specific cause of these growth defects is currently unknown but may result from problems with chromosome segregation and genome stability, as observed for *S. pombe* and *D. melanogaster* strains that harbor mutations in their respective DIM-5 homologs (Grewal and Jia, 2007; Peng and Karpen, 2008). In *N. crassa*, tandem repeats of transforming DNA have been reported to be unstable in a *dim-5* mutant, suggesting that H3 K9 methylation may serve to prevent mitotic recombination between repeated sequences (Chicas et al., 2005).

DIM-5 catalyzes mono-, di-, and trimethylation of H3 K9 in vitro, but trimethyl H3 K9 is the predominant product, both in vitro and in vivo (Tamaru et al., 2003; Zhang et al., 2003). In *S. pombe* and animals, methylated H3 K9 is recognized and bound by the chromo domain protein HP1 (Bannister et al., 2001; Grewal and Jia, 2007; Lachner et al., 2001). *N. crassa* encodes a single HP1 homolog, which is required for DNA methylation (Freitag et al., 2004). Cytologically, HP1 localizes to three or four distinct heterochromatic foci in wild-type cells, and proper localization requires DIM-5. While the chromo domain of HP1 directly binds H3 methylated at K9, a C-terminal chromo shadow domain interacts directly with a tandem pair of PXVXL-related motifs found in the N-terminal domain of DIM-2 (Freitag et al., 2004; Honda and Selker, 2008). Thus, HP1 serves to link methylated H3 K9 and DNA methylation. High-resolution analysis of the distribution of trimethyl H3 K9 and HP1 across LGVII revealed that both exhibit extensive colocalization with DNA methylation (Fig. 2) (Lewis et al., 2009). All regions that were enriched for trimethyl H3 K9 were enriched for DNA methylation, indicating that methylation of H3 K9 is sufficient to trigger DNA methylation. HP1 associates with domains of H3 K9 methylation but exhibits a complex localization pattern, indicating that dynamic binding of HP1 or accessory proteins contribute to the steady-state distribution of the protein. Notably, the distribution of trimethyl H3 K9 is largely unaffected in mutants that lack DNA methylation or HP1. Therefore, the pathway for heterochromatin formation appears to be largely unidirectional in *N. crassa*. In addition, DNA methylation was restored at all LGVII heterochromatic domains following depletion and subsequent reintroduction of the H3 K9 methylation machinery. Together, these data suggest that RIP'd DNA is sufficient to direct H3 K9 methylation de novo and that heterochromatic domains in *N. crassa* are defined molecularly by RIP'd DNA, H3 K9 methylation, HP1, and DNA methylation.

Although the molecular events downstream of H3 K9 methylation are becoming clear, the signals that direct DIM-5 to regions of RIP'd DNA are not yet well defined. In *S. pombe*, the RNA interference (RNAi) pathway is involved in the establishment of H3 K9 methylation by Clr4 at pericentric heterochromatin and works in concert with other pathways to target K9 methylation at the silent mating type locus (Grewal and Jia, 2007). Similarly, components of the RNAi machinery are important for some DNA methylation in plants (Henderson and Jacobsen, 2007). In *N. crassa*, however, the RNAi pathway is not involved in maintenance or de novo DNA methylation (Borkovich et al., 2004; Chicas et al., 2005; Freitag et al., 2004). Also, H3 K9 methylation and HP1 localization do not require the RNAi pathway.

Treatment of *N. crassa* with the HDAC inhibitor TSA leads to loss of some DNA methylation, indicating that HDAC activity is required for DNA methylation of some, but not all methylated regions in this fungus (Selker, 1998). Indeed, two of the four acetyl coenzyme A-dependent HDACs of *N. crassa* are required for normal DNA methylation (K. M. Smith, J. Dobosy, G. R. Green, D. A. Anderson, and E. U. Selker, unpublished data). It will be important to identify the protein(s) that recognize RIP'd DNA to gain a complete understanding of heterochromatin formation in *N. crassa*.

In *A. immersus*, DNA methylation is associated with dimethyl H3 K9 rather than trimethyl K9 (Barra et al., 2005). The enzyme that performs this modification has not been identified. In addition, it is not known if DNA methylation can direct K9 methylation in this fungus. H3 K9 methylation has also been described in *Aspergillus fumigatus*, a filamentous fungus that lacks DNA methylation (Palmer et al., 2008). *A. fumigatus* strains that lack ClrD, a homolog of DIM-5, display defects in growth and development, similar to *N. crassa dim-5* mutants. In addition, *clrD* mutants display increased sensitivity to 6-azauracil, suggesting that transcription is impaired in these strains. Interestingly, *clrD* mutants show reduced mono- and trimethyl H3 K9, whereas dimethyl K9 levels appear to be unaffected, suggesting that multiple H3 K9 methyltransferases are present in *A. fumigatus*. A complete understanding of H3 K9 methylation in the fungi awaits more-detailed analyses of the genomic localization and the proteins responsible for this modification.

CONCLUSIONS

The investigation of chromatin structure and function in the filamentous fungi has been fruitful, but much remains unknown. Important functions for chromatin in transcription, gene silencing, and genome defense have been uncovered. It will be interesting to determine how histone variants, chromatin remodeling, and chromatin modifications impact additional biological processes in filamentous fungi. For example, the role of chromatin structure in DNA repair and recombination is largely unknown for this group. Also, it will be interesting to determine if changes in chromatin structure accompany development or pathogenesis. The complete genome sequences available for many fungi allow high-resolution mapping of the distributions of modified histones, histone variants, and nonhistone chromatin proteins, which should provide some clues to their functions. Future genetic, biochemical, and molecular studies are necessary as well. Together, these analyses will provide a more complete understanding of chromatin structure and function in filamentous fungi.

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10

Transposable Elements and Repeat-Induced Point Mutation

A. JOHN CLUTTERBUCK AND ERIC U. SELKER

Fungi have relatively compact genomes. While a plant or animal may contain 30,000 genes in a genome of about 4,000 Mb, a typical filamentous fungus contains about 10,000 genes in a haploid genome of 40 Mb or less. The compact nature of fungal genomes probably reflects selection for small nuclear and cell volumes, compatible with wind-dispersed asexual spores, narrow hyphae, and in some cases passage of nuclei through septal pores. Not surprisingly, fungi appear to be adept at controlling "selfish DNA," such as transposable elements (TEs). In most fungi examined, TEs comprise no more than 3% of the genome, implying effective TE repression and destruction mechanisms. Touchon and Rocha (2007) found that genome size in bacteria was the main factor correlating with insertion sequence abundance. The high TE content of *Laccaria bicolor* (Martin et al., 2008), accompanying a genome approximately twice the size of that in most filamentous fungi, and the paucity of TEs in *Saccharomyces cerevisiae* suggest that this correlation applies to fungi as well.

The first discovered, and probably most potent, genome defense mechanism is called repeat-induced point mutation (RIP). Although RIP was initially found in the filamentous fungus *Neurospora crassa*, other fungi show evidence of a mechanism similar or equivalent to RIP, as discussed in this chapter. RIP in *Neurospora* is first briefly described, and then, what is known about the distribution and condition of TEs in this and other filamentous fungi is summarized. Recent reviews (Daboussi and Capy, 2003; de Lima Fávaro et al., 2005) have given broad coverage to many aspects of TEs in filamentous fungi, including their use as analytical tools and in population or taxonomic studies, which are not updated here. Instead, this chapter concentrates on the impact of RIP on TEs.

RIP

RIP was discovered as a result of a detailed analysis of progeny from crosses of *N. crassa* transformants (Selker, 1990). It was found that duplicated sequences, whether native or foreign, and whether linked or unlinked, were frequently subjected to numerous polarized transition mutations (GC to AT) in the haploid genomes of the special premeiotic binucleate cells of the ascogenous tissue resulting from fertilization (Fig. 1) (Cambareri et al., 1989, 1991; Foss et al., 1991; Selker et al., 1987; Selker and Garrett, 1988). Experiments in which the stability of a gene was tested when it was unique in the genome or else combined with an unlinked copy demonstrated that RIP is not simply repeat *associated*; it is clearly repeat *induced* (Selker and Garrett, 1988). In a single passage through the sexual cycle, up to ~30% of the GC pairs in duplicated sequences can be mutated in *N. crassa* (Cambareri et al., 1989, 1991). A likely mechanism for generation of C→T transitions is methylation of cytosines followed by spontaneous or enzymatic deamination and DNA replication (Selker, 1990). The putative deamination step might be catalyzed by a DNA methyltransferase (DMT) or similar enzyme. Consistent with this possibility, one of two putative DMTs predicted from the *N. crassa* genome sequence is involved in RIP (Freitag et al., 2002). Progeny from homozygous crosses of mutants with defects in this gene, *rid* (RIP defective), do not show RIP activity. Mutants defective in *rid* do not have any obvious defects in DNA methylation in vegetative hyphae or in fertility, growth, or development. While methylation has not been documented in the sexual phase of the life cycle, most sequences affected by RIP are found to be methylated in vegetative hyphae. The *Neurospora* DMT DIM-2, which was identified genetically (Foss et al., 1993), is necessary for vegetative DNA methylation but is not required for RIP (Kouzminova and Selker, 2001).

All indications are that every sizable duplication (greater than ~400 bp for tandem duplication or ~1,000 bp for unlinked duplication) in *Neurospora* is susceptible to RIP. Nevertheless, duplications escape RIP at some frequency (typically less than 1% in one passage through the sexual cycle for a tandem duplication or approximately 50% for an unlinked duplication). Even duplications of chromosomal segments containing numerous genes are sensitive to RIP (Bhat and Kasbekar, 2001; Perkins et al.,

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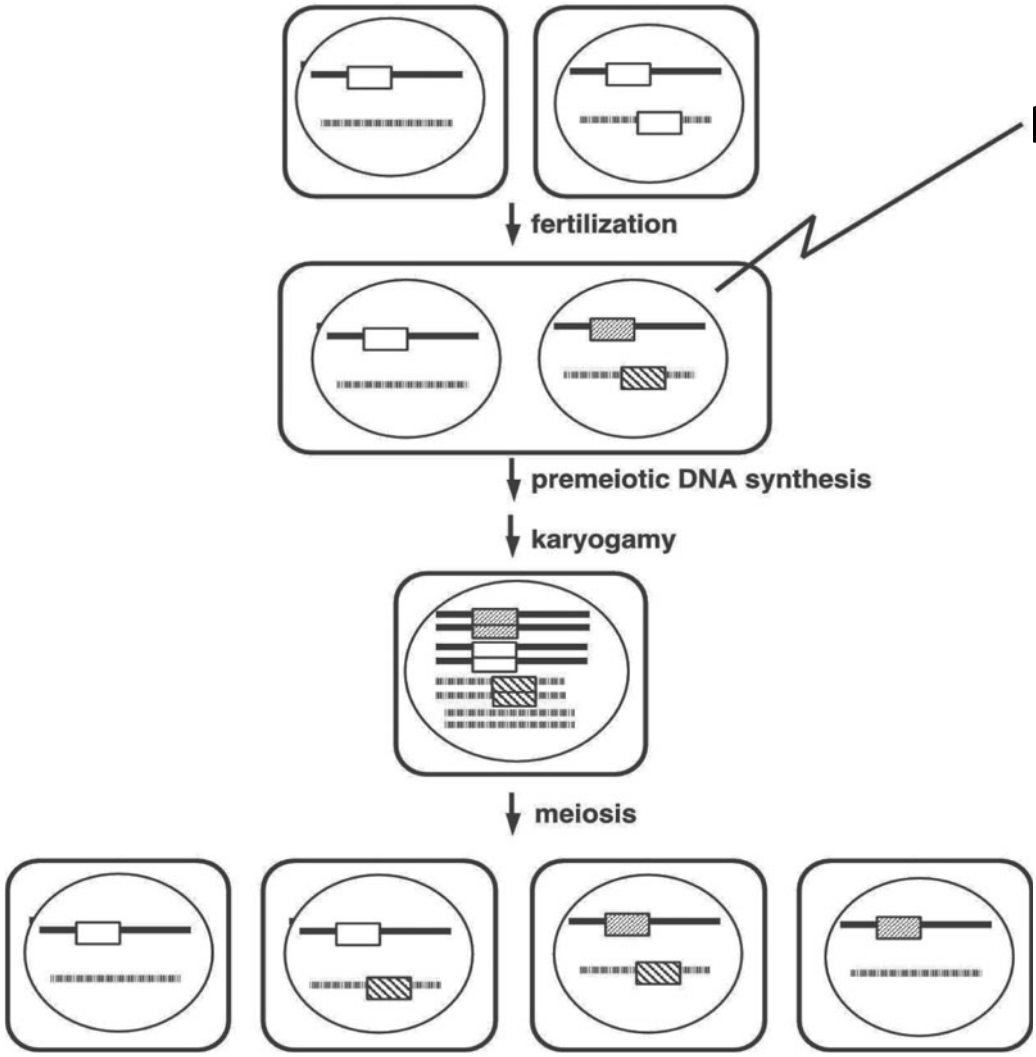

FIGURE 1 Diagram of RIP in *Neurospora*. Two haploid strains of opposite mating type are illustrated, one containing an unlinked duplication (open boxes). For clarity, only two chromosomes are shown. The lightning bolt indicates the time that RIP occurs, and the filled boxes indicate new alleles created by RIP. The four possible combinations of chromosomes in the meiotic progeny are represented below. In cases in which an essential gene was duplicated, only the left-hand two products would be expected to be viable. Unlinked gene-sized duplications pass through a cross without being discovered and mutated by RIP at an appreciable frequency (~50%), but direct duplications of the same sequences rarely survive untouched. Most products of RIP are left methylated at remaining cytosines.

1997). The existence of RIP has raised the question of whether *Neurospora* can utilize gene duplications to evolve. The *N. crassa* genome sequence revealed gene families, but tellingly, virtually all paralogues were found to be sufficiently divergent that they should not trigger RIP (Galagan and Selker, 2004). Exceptions include the rRNA gene cluster and genes with relatively short regions of similarity, such as an unlinked pair of histone H4 genes. Thus, RIP may indeed limit evolution in *Neurospora* (Galagan and Selker, 2004).

RIP has been demonstrated experimentally, using multicopy transgenes, in a few other filamentous ascomycetes: *Podospora anserina* (Graia et al., 2001; Bouhouche et al., 2004; Arnaise et al., 2008), *Magnaporthe oryzae* (*M. grisea*) (Ikeda et al., 2002), and *Leptosphaeria maculans* (Idnurm and Howlett, 2003), but no evidence of RIP was found in *Sordaria macrospora* (Le Chevanton et al., 1989; Walz and Kück, 1995). It is clear that RIP does not follow an identical pattern in all fungi: whereas RIP in *N. crassa* is intense enough to reduce the C+G content of the most affected elements to below 30% and widespread enough for unmutated TEs to be absent from the sequenced genome (Galagan et al., 2003), in *M. grisea* and *P. anserina* RIP is described as "light" or "mild" (Ikeda et al., 2002; Graia et al., 2001), and in *P. anserina* it was observed only in late-maturing ascospores. In both *N. crassa* and *P. anserina*, the majority of mutated cytosines were found in CpA dinucleotides, but in *M. grisea*, the preferred context for RIP was (A/T)pCp(A/T).

Interestingly, some fungi show what appear to be milder genome defense systems that are similar to RIP. The most

notable example is MIP (methylation induced premeiotically) in *Ascobolus immersus*, a process that, like RIP, detects linked and unlinked sequence duplications during the period between fertilization and karyogamy, but relies exclusively on DNA methylation for inactivation (Barry et al., 1993; Faugeron et al., 1990; Goyon et al., 1996; Goyon and Faugeron, 1989; Malagnac et al., 1999; Rhounim et al., 1992; Rossignol and Faugeron, 1994). The sensitivity of MIP in *Ascobolus* to tandem and unlinked duplications appears to be equivalent to that of RIP in *Neurospora* (Goyon et al., 1996).

DNA methylation in vegetative mycelia has been reported for apparent relics of RIP in *M. grisea* (Ikeda et al., 2002) and *P. anserina* (Graia et al., 2001), as in *N. crassa*, but was not found in *L. maculans* (Idnurm and Howlett, 2003), and no more than trace quantities of 5-methylcytosine have been found in *Aspergillus* species (Antequera et al., 1984; Gowher et al., 2001).

TEs IN FILAMENTOUS FUNGI

The genomes of *Neurospora* and other filamentous fungi show a large variety of sequences homologous to TEs found in other organisms. In a 2003 review of TEs in filamentous fungi, Daboussi and Capy (2003) list 67 individually named elements, to which many more could now be added. These include two new categories of class II DNA transposons: *Helitrons* and *Polintons* (Kapitonov and Jurka, 2001, 2006). Most fungal elements have been discovered accidentally or by searching for elements of a particular class. The discovery of TE sequences in *Neurospora* was facilitated by the fact that nearly all of them have been subjected to RIP and are found methylated in the vegetative mycelium. In a preliminary characterization of methylated sequences in *N. crassa*, Selker et al. (2003) compiled a list of 32 named TEs, as well as some unidentified fragments. Many more relics of RIP were discovered when the whole genome was sequenced (Galagan et al., 2003). In *M. grisea*, the distribution of members of 13 TE families was studied in bacterial artificial chromosome end sequences (Thon et al., 2004). A search for TEs in the large (65-Mb) genome of the mycorrhizal basidiomycete *L. bicolor*, using a variety of techniques, revealed that 21% of the genome is accounted for by such elements (Martin et al., 2008). Similarly, Clutterbuck et al. (2008) reported systematic surveys for TEs by comparing repetitive sequences in the genomes of *Aspergillus nidulans*, *Aspergillus fumigatus*, and *Aspergillus oryzae* (Galagan et al., 2005) with known elements listed in Repbase (Jurka et al., 2005). The wealth of elements found makes it impractical to give them individual names, so new elements are named by their superfamily, a family number, and species abbreviation, e.g., the *A. fumigatus* element *Mariner-6_AF*. More recently Wicker et al. (2007) have proposed a unified classification and code-naming system for all TEs.

Most filamentous fungal genomes surveyed contain a wide variety of elements from known TE superfamilies, each fungus containing its own unique set of elements. Among class I retrotransposons, *Gypsy*-superfamily long-terminal-repeat (LTR) elements are common, and many species contain more than one such family. *Copia* superfamily LTR elements also occur in many species. All *Copia* elements in *A. nidulans* and *A. fumigatus* are degenerate (Clutterbuck et al., 2008), and while it has been suggested that *Copia* elements are underrepresented in filamentous fungi (de Lima Fávaro et al., 2005), they are abundant in *Microbiotrium violaceum* (Hood et al., 2005), *L. bicolor* (Martin et al., 2008), and *Aspergillus niger* strain CBS513.88 and its derivatives (Braumann et al., 2007). *LINE*-like, non-LTR retrotransposons, e.g., "*I*" elements, are also common.

Class II DNA transposons are most frequently represented by multiple *Mariner* families of *Pogo* and *Tc1/Mariner* clades; *MUDR (MULE)*, *hAT*, *Helitron*, and *Polinton* DNA elements are also found in some species. *Mariner* elements generally encode only a single transposase, but some "*long mariners*" found in *A. fumigatus* and *A. oryzae* are distinguished by the presence of one or more additional open reading frames (Galagan et al., 2005). The *A. nidulans* genome includes four putative *MUDR* families, one of these being the "*MATE*" (*DNA-3_AN*) element (Aleksenko and Clutterbuck, 1997), fragments of which confer autonomous replication on AMA1 plasmids in *Aspergillus* and *Penicillium*. The basis of this phenomenon is unknown, but the ability of this element to enable plasmid replication suggests the occurrence of a replicative stage in the parent transposon. *Helitron* elements are characterized by an encoded helicase that is believed to function in replication by a rolling-circle mechanism (Kapitonov and Jurka, 2001). They were found in the *A. nidulans* genome (Galagan et al., 2005; Clutterbuck et al., 2008) and homologues of the *A. nidulans* element are abundant in *Neosartorya fischeri*, which is noted for its relatively high TE content (Fedorova et al., 2008). The *L. bicolor* genome also contains many *Helitron* fragments (Martin et al., 2008). *Polintons* may contain multiple genes, characteristically including DNA polymerase B (Kapitonov and Jurka, 2006). Representatives were found in two fungi: the soybean rust *Phakopsora pachyrhizi* and the arbuscular mycorrhizal fungus *Glomus intraradices*.

Intact elements with full open reading frames, e.g., the 15 near-identical copies of *Mariner-6_AN* (the largest *Mariner* family in Fig. 2A), can be considered to be potentially mobile. Most degenerate elements are probably immobile. However, if abundant copies of similarly deleted elements are found, this suggests that these are nonautonomous elements that have lost their transposase functions but can spread passively using the transposases of related, intact elements. For example, *A. nidulans* contains two families comprising 192 nonautonomous *hATN* elements (Fig. 2B), although only two fragments of intact *hAT* elements remain in the genome. Other examples of nonautonomous elements occur in many classes, the most prominent being *SINEs* (related to *LINEs*) and *MITEs* (deleted *Mariners*). The *A. oryzae* genome contains *SINE* elements with homology to the 5′ half of 5S rRNA, which appear to be new to fungi but are also present as fragments in other aspergilli (Galagan et al., 2005). The origin of a nonautonomous *Helitron-N1_AN* by deletion and adoption of a new 3′ end has been traced in detail (Cultrone et al., 2007).

Another distinctive class of TE fragments consists of solo LTRs, presumed to result from excision of the internal sequences of LTR retrotransposons by recombination between their terminal repeats. The frequency of such solo elements may reflect the recombination potential of their host genome. Interestingly, the homothallic species *A. nidulans* contains 505 solo LTRs from known or unknown retroelements (Fig. 2B), while *A. fumigatus* and *A. oryzae* have only 150 and 186, respectively, although all these species have numerous LTR retrotransposons (Clutterbuck et al., 2008). Since no fruiting bodies were observed, these fungi have long been assumed to be asexual, but the recent discovery of heterothallic sexual reproduction in *A.*

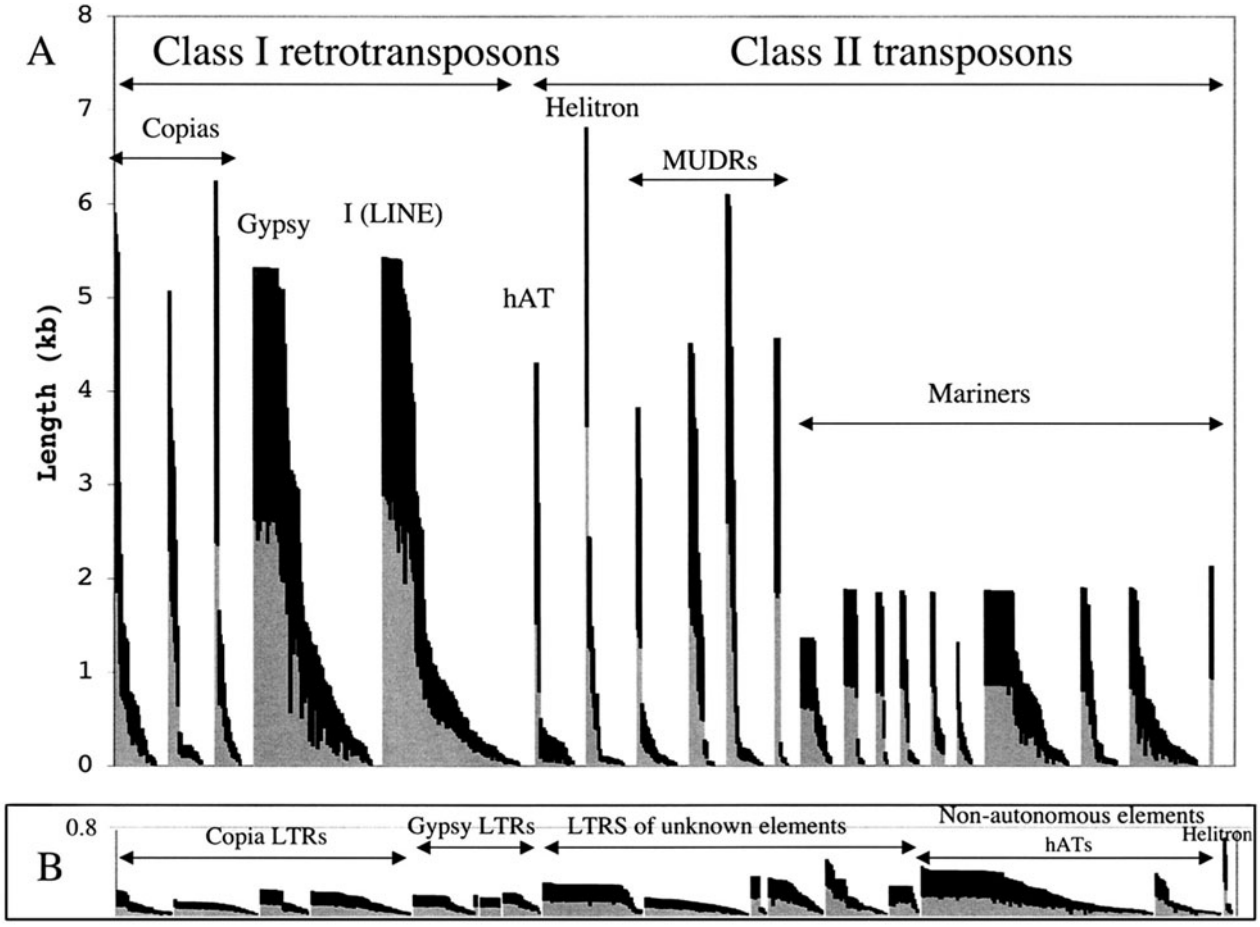

FIGURE 2 Graphic list of TEs in *A. nidulans*. Each vertical line represents one element, elements being ranked by length within families. In each element the black, upper portion of the line represents A+T content while the gray lower part represents C+G; the predominance of A+T in some elements is suggestive of the action of RIP. (A) Long elements and their fragments. (B) Small elements, including solo LTRs and nonautonomous elements.

fumigatus (O'Gorman et al., 2009) puts this assumption in doubt for other "asexual" fungi.

GENOMIC DISTRIBUTION OF TES

The distribution of TEs in fungal genomes suggests that there are a limited number of innocuous genomic locations. TEs can damage their hosts by insertion into functional coding or regulatory sequences; Daboussi and Capy (2003) list numerous instances of this, the resulting mutations often being the first evidence of the existence of a novel TE. They also cause genome rearrangement by homologous recombination between unlinked elements of the same family. It has been noted that chromosomal rearrangement breakpoints in the lineages of the three *Aspergillus* species analyzed often coincide with regions of TE clusters (Galagan et al., 2005). Similarly, blocks of conserved synteny between *M. oryzae* (a member of the *M. grisea* complex) and *N. crassa* are separated by TE clusters in *M. oryzae* (Thon et al., 2006). Aberrant excision, involving interaction between two copies of an element, as described by Hua-Van et al. (2002), can cause local chromosome rearrangements. Comparative synteny maps of three aspergilli (Galagan et al., 2005) suggest that this has been a common evolutionary event and could explain the puzzling reshuffling, without dispersal, of gene clusters, e.g., of the quinate catabolism genes (Giles et al., 1991; see also chapter 26).

Centromere sequences fail to compile in most genome projects because they consist of highly repetitive material, and contigs abutting centromeres generally end in a cluster of very AT-rich TE fragments, although adjacent regions of *A. nidulans* are mostly TE-free. Telomeres may also be excluded from fungal genomes sequences, although in this case it is because the terminal contigs are too short to be accepted by the compiler (Clutterbuck and Farman, 2008). Clustered relics of TEs are frequent within 100 kb of telomeres, but the short subtelomeric domains in *A. nidulans* are TE-free (Clutterbuck and Farman, 2008). It seems likely that these segments, although variable and probably subject to RIP, cannot be interrupted without damage to the host. It is noteworthy in this context, however, that extensive segments of fungal genomes appear to be nonessential

(Takehashi et al., 2006). Connelly and Arst (1991) described a viable deletion of what is now known to be a 270-kb telomeric segment of *A. nidulans* chromosome III containing 10 TE fragments and 96 putative (autocalled) genes. Thus, primary TE insertion sites may often be in nonessential distal regions of chromosomes, but other TE cluster regions could be the result of chromosomal rearrangements in which TEs have participated.

Of the three *Aspergillus* species analyzed by Clutterbuck et al. (2008), *A. fumigatus* has the most numerous full-length TEs, possibly correlated with the lowest level of genome rearrangement in its ancestry (Galagan et al., 2005). Fifty-five percent of TEs in this species are found in tight clusters, an appreciable proportion of which can be interpreted as the result of "nesting," i.e., the invasion of resident elements by new ones. In virtually all these cases, the invaded element has reduced C+G content compared to the invader. The commonest of these invaders in *A. fumigatus* are *Gypsy-1_AF* elements, and analysis of flanking regions of nonnested, i.e., scattered, *Gypsy-1_AF* insertions gives some support to the hypothesis that they preferentially insert into AT-rich areas, since in all cases the flanking 100 bp has an A+T content of >50% and in most cases >60%.

Despite the prominence of TE clusters, 45% of *A. fumigatus* elements are scattered throughout the genome. Typical of these are *Mariner* elements, many of which are young, full-length copies that have evidently managed to insert between genes without damage to the host. Li Destri Nicosia et al. (2001) reported a failure to find any cases of TE insertion as a cause of spontaneous mutation in the *A. nidulans niaD* gene. This suggests that all *A. nidulans* elements are very effectively repressed or that TEs have evolved to avoid insertion into active genes. Evidence of TE target site choice has been found mainly for eukaryote retroelements; this was reviewed by Bushman (2003) and earlier by Craig (1997). The *S. cerevisiae Ty3* element represents a classic example, in which this element preferably inserts close to polymerase III transcription initiation sites (Chalker and Sandmeyer, 1992). A related recent investigation (Dai et al., 2007) revealed that phosphorylation directs the integration of *Ty5* into heterochromatin. A precise target site preference is also shown by the majority of intact copies of the *LINE* element *I-1_AF* in *A. fumigatus*, which are inserted at the same site in the LTRs of *Afut2_AF* elements (Galagan et al., 2005; Clutterbuck et al., 2008).

LIFE AND DEATH OF TEs

The origins of novel TEs are generally unknown, but evolution of LTR retrotransposons can often be traced within the host organism. Class II DNA transposons more commonly fall into discrete families of near-identical elements. Population studies regularly show variation in TE content or location between isolates, and reservoirs of active elements may exist within the species; a good example of this is the finding of an active *Neurospora Tad* element in an African population, when laboratory strains and other wild isolates contained only defective remains (Kinsey and Haber, 1989). Another example is the *Copia*-like *ANiTa1* element found in the genome of *A. niger* industrial strain CBS513.88 and its derivatives (Braumann et al., 2007). In these strains most *ANiTa1* copies are young in appearance, but none with intact internal sequences are found in the ATCC 1015 strain sequenced by the Broad Institute. The presence of members of the same TE superfamilies in a wide range of plants, animals, and fungi implies that horizontal gene transfer must have occurred, and while suggestive evidence of this is abundant, final proof is rare (Daboussi and Capy, 2003).

Daboussi and Capy (2003) listed a considerable number of TEs that have demonstrated mobility. A recent addition to this list is the *Aft1* (*Mariner-1_AF* in Repbase) element of *A. fumigatus* (Hey et al., 2008). Mobility in the wild is indicated by *Aft1* site polymorphism in *A. fumigatus* strains, and in the laboratory, evidence of mobility consists of *Aft1* excisions from a plasmid reintroduced into its parent strain. The rarity of such events contrasts with the proliferation of *Fusarium Fot1* elements introduced into a naïve *Aspergillus* host (Li Destri Nicosia et al., 2001). Similarly, Ikeda et al. (2002) introduced the *M. grisea MAGGY* element into a strain that did not previously contain it, resulting in the generation of 15 to 20 copies in different progeny strains derived from a single transformant.

The pattern of bursts of activity for new elements, to give a limited number of copies that are then apparently repressed, suggests copy-number-dependent, family-specific autorepression. A number of repression systems are partially understood (for reviews, see Slotkin and Martienssen, 2007, and Maxwell and Curcio, 2007; see also chapters 9, 11, and 12). One remarkable mechanism for repression of class I LTR retrotransposons reported in *Schizosaccharomyces pombe* employs a CENP-B centromere binding protein homologue, apparently derived from the transposase of a class II *Mariner* element (Cam et al., 2008). The *S. pombe* CENP-B homologues act by aggregating their target sites at the ends of LTR retrotransposons into heterochromatic clusters. It appears that "domestication" (or "exaption") of *Mariner* transposase for this function has occurred separately in *S. pombe* and mammals but has not occurred in other organisms (Casola et al., 2008).

Stress may cause release from repression and allow resumption of transposition (Daboussi and Capy, 2003). A recent example from fungi is the heat shock activation of DNA transposons in *Ophiostoma ulmi* (Bouvet et al., 2007). Transposition activation by stress can be seen as a panic measure: from the perspective of the element, it might lead to release of free elements potentially available for horizontal transfer to a safer host. From the perspective of the host organism, transposon activation can lead to random chromosomal rearrangements, and it has recently been demonstrated that a proportion of stress-induced rearrangements, although not shown to be related to TE activity, may confer resilience to the initial stress (Coyle and Kroll, 2008).

Older TE families have evidently suffered from destruction (e.g., by RIP) as well as repression. In addition, Fig. 2 shows that a large proportion of recognizable elements are fragmented; aberrant transposition is one TE-specific mechanism of fragmentation (Hua-Van et al., 2002), but at present there is little indication that the majority of fragmentation is the result of anything other than the destructive processes suffered by the rest of the genome.

EXTENT OF DAMAGE OF TEs BY RIP

A striking feature of some of the TE sequences illustrated in Fig. 2A is their low C+G content, presumably due to the action of RIP. Daboussi and Capy (2003) list a number of fungi in which RIP has been either observed experimentally or deduced from the presence of numerous C→T transitions in repeated genomic elements. Sequences that resemble known relics of RIP have been reported in a variety of filamentous ascomycetes, including *A. nidulans*, *A. fumigatus*, and *A. oryzae* (Clutterbuck, 2004; Galagan et al., 2005; Montiel et al., 2006; Hey et al., 2008; Clutterbuck et al., 2008), *A. niger*

and *Penicillium chrysogenum* (Braumann et al., 2007), and *Colletotrichum cereale* (Crouch et al., 2008). Depletion of C+G content of repetitive genomic sequences can best be followed where a number of near-identical, undamaged copies of the element can be used to compile a consensus, with which other, C+G-depleted copies, can be compared. *"MATE"* (*DNA-3_AN*) elements (Clutterbuck, 2004) are represented by two nearly identical 6.1-kb copies, a third incompletely sequenced copy, and two more copies that differ individually from the consensus by a few transversions and 263 and 306 C→T transitions respectively (counting replacements on both strands). This suggests that RIP has operated here.

A survey of TE samples in 28 filamentous ascomycete genomes, backed up by genome scans for C+G-depleted tracts (A. J. Clutterbuck, unpublished data), shows that C+G depletion is subphylum-wide. Of course, genomic data do not tell us whether the mechanisms involved are specific to repeats, at what stage in the life cycle they occur, nor whether the process is still active in any species. The data do, however, show that depletion can be more extreme than is apparent from experimental studies of RIP. The resulting heavily mutated sequences will usually be unrecognizable as TEs, but in some cases a consensus sequence can still be constructed, for example, for the *A. oryzae Mariner1-AO* element, which shows C+G content of just 17.5% (Clutterbuck et al., 2008). It has been noted that the *A. oryzae* genome includes numerous stretches of DNA with A+T content of >90% (Machida et al., 2005). This is a statistic that varies from 0.001 to 2.2% in the species surveyed, with *N. crassa* and *A. nidulans* being near the high and low ends of the range, at 0.5 and 0.04%, respectively. The preferred context for C→T transitions also appears to vary; while CpA dinucleotides are the most frequently affected in *N. crassa* and its relatives, CpG dinucleotides appear to be most commonly mutated in many species, and the central tracts of degraded TEs can be completely devoid of this dinucleotide (Clutterbuck, unpublished). Cytosines flanked on the 3′ side by pyrimidines are generally less affected than those adjacent to purines. In *A. nidulans* and *A. fumigatus*, CpY dinucleotides are hardly diminished at all, while in other species either CpC or CpT dinucleotides approach CpR dinucleotides in depletion frequency.

Homologues of the *N. crassa rid* gene are present in all *Aspergillus* species whose genomes have been sequenced to date, as well as in other filamentous fungi (Galagan and Selker, 2004). The *rid* homologue in *A. nidulans*, *dmtA*, is essential for completion of sexual reproduction (Lee et al., 2008), although, like *rid* in *Neurospora*, its deletion does not affect vegetative growth. Consistent with the expectation that RIP would only occur premeiotically, as in *N. crassa* (Fig. 1), Ikeda et al. (2002) found that high levels of RIP in *M. grisea* were found only in isolates that were sexually fertile under laboratory conditions. The sexual status of many ascomycetes is unknown, but the absence of sexuality cannot be assumed from failure to observe it. Many studies, e.g., on *Penicillium* and *Aspergillus* species (LoBuglio et al., 1993; Geiser et al., 1996) indicate that sexual and apparently asexual species are closely related. The facility to detect mating-type genes in wild populations, including the determination of homothallic or heterothallic status, and the distribution of opposite mating types in heterothallics (O'Gorman et al., 2009, plus supplementary information online [see O'Gorman et al., 2009]), can be expected to lead to increased detection of sexuality. While the frequency of sexual reproduction, or indeed the time since it last occurred in a species, may be important for the observation of C+G depletion in TEs, it is also clear that the extent of such observation will depend greatly on the quantity and age of TEs in each species, i.e., on the unique history of TE incursion and proliferation in each species.

FINAL COMMENTS

TEs represent a genome component that, with rare exceptions, is of no value to the host organism and can be detrimental. Like other genomic components, TEs provide a record of the evolutionary processes to which they were subjected, but unlike most genomic components, their turnover is rapid and the record they leave is largely one of decay, including defeat by the defense mechanisms of the host. RIP is the most potent genome defense system known in eukaryotes. It is perhaps surprising that RIP is apparently unique to filamentous ascomycetes, but genome defense mechanisms appear to be unusually labile in evolution (for an example, see Hammond et al., 2008; see also chapter 12), perhaps because they have to be retailored to meet each new emergency encountered by the host. Moreover, RIP carries costs for the host, the most significant of which is probably that it limits the evolution of gene families.

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11

Meiotic *trans*-Sensing and Silencing in *Neurospora*

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Sexual reproduction is good for organisms and can be beneficial for the propagation of transposable elements (TEs) that could use this developmental stage to "jump" and "infect" pristine genomes. While the mobilization of the elements themselves is mutagenic, their abundant presence in genomes of sexual, especially Metazoan organisms, suggests either that their presence brings benefits or that removing them is just too costly for the organism. Evidence suggests that in fact TEs may not always be detrimental, as they probably contribute to aspects of genetic diversity and/or chromosome stability (Pardue and DeBaryshe, 2003). This widely accepted dogma states that the genomic landscape is shaped largely by the ongoing battles waged between genetic programs contributing "constructively" to the stable inheritance of a species' DNA and the exquisitely regulated expression of its genes and the invasive, parasitic genetic elements such as transposons (Hickey, 1993). We think it is reasonable to say that these past battles largely shaped the genome of many organisms including that of *Neurospora crassa*, an organism that exhibits a very rigorous and diverse set of genome defense programs. Studying the biology of these genome defense mechanisms in *Neurospora* will certainly help us understand how different organisms dealt with the evolutionary forces aiming at shaping the different genome's architecture through time.

At the center stage of all sexual reproduction is meiosis, a complex molecular process during which the controlled genetic exchange of genes and gene segments between genomes of organisms belonging to the same species occurs. From an engineering point of view, meiosis places a set of paternal and maternal chromosomes into the same cellular compartment and then proceeds to break down their gene combinations into smaller parts, recombining and reassembling them into new, possibly never before seen chromosome combinations. The genome products of meiosis now have the ability to reconstitute metabolic pathways, organelles, tissues, and organs by using their newly acquired gene assemblies. This process has profound implications especially for complex adaptation traits and phenotypes produced and composed of a great many different genes. A simple explanation of why meiosis has proven to be nondeleterious to the maintenance of these complex processes might be that, despite its ability to literally shred the genome into pieces, it might also have developed molecular mechanisms that ensure that the basic pieces that constitute the different pathways and/or processes are present on both genomes. What is likely to be a primordial form of such checkpoint mechanisms is exemplified by meiotic *trans*-sensing and silencing, processes found and discovered in *N. crassa*.

Before delving into the complexities of meiotic silencing, we think it is important to remind the reader that before any genetic exchange can take place between the chromosomes participating in meiosis, these molecules must first physically pair. The term "pairing" has, unfortunately, different meanings. To make things worse, the term is sometimes used interchangeably with "synapsis," a word that has a more restricted meaning. We thus find it necessary to define these terms. We find at least four different kinds of chromosome "pairing" in meiosis (Cook, 1997). First is the cohesin-dependent pairing that results from the duplication of the strands of DNA after premeiotic DNA replication. Second is the long-distance pairing or "alignment or coalignment or juxtaposition" that is observed at leptotene, where each duplicated pair searches for its (duplicated) homolog. Third is the short-distance pairing or synapsis that is seen at zygotene, where the building of the synaptonemal complex draws the homologs even closer together so that by pachytene they lie in close register with one another. Fourth is the single-stranded DNA (ssDNA)-ssDNA complementary pairing or "complementary base-pair pairing" that precedes the recombination that takes place between two DNA duplexes that must lie within nanometers of each other.

In general, our understanding of all homology-sensing mechanisms is rather poor. So is our understanding of the connections between the meiotic long-distance pairing, meiotic *trans*-sensing, and recombination. The process of meiotic long-distance pairing must involve *trans*-sensing between chromosomal segments, but the relationship between these kinds of sensing and that of the *trans*-sensing that activates meiotic silencing is unknown. For the sake of simplicity, we therefore consider the *trans*-sensing that

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is involved in the early evaluation of every chromosomal DNA segment and the one involved in meiotic silencing as being the same. The *trans*-sensing between the genomes participating in meiosis occurs concomitantly with the coalignment of homologous chromosomes and requires extensive searching and satisfaction of stringent molecular homology criteria before recombination is allowed. The perceived absence of such an equivalent DNA segment as "sensed" by this meiotic "*trans*-sensing" mechanism triggers in many organisms the establishment of unique chromatin architectures such as those mediated by histone modifications and specialized nonhistone proteins early in meiosis (Ivanovska and Orr-Weaver, 2006; Prieto et al., 2004). In *Neurospora*, the lack of a homologous region triggers, we think, the destruction of RNA molecules with homology to the "nonhomologous" region. This uses core components of the RNA silencing pathways found in other organisms. At the molecular level the only well-understood homology-sensing process is recombination, a process whose final product appears relatively late in meiosis. Importantly, not only does homology sensing play a critical role in blocking ectopic recombination, but also its absence can clearly have catastrophic consequences for the organism (Bourc'his and Bestor, 2004). Perhaps it is therefore not surprising to find that the same molecular mechanisms used by many types of cells to recognize and suppress viruses, retrotransposons, and insertion sequences have been either adapted from, or adapted to, meiotic mechanisms optimized to maintaining genome integrity. One such example is meiotic silencing.

A WORD ABOUT *NEUROSPORA* BIOLOGY

Understanding meiotic silencing in *Neurospora* requires knowledge about the life cycle of *N. crassa* (reviewed by Davis and de Serres, 1970) (Fig. 1). *N. crassa* is a heterothallic filamentous fungus with two nonswitching mating types, *A* and *a*. It grows vegetatively as a multinucleate mass of interconnected hyphal cells called mycelia. Asexual reproductive development involves the production of aerial hyphae from which conidiophores form chains of terminal asexual spores called conidia. Conidia can germinate to form a new mycelial network, thus completing the vegetative cycle. Under poor nitrogen and low-temperature (i.e., 25°C) conditions, sexual development is activated. From these sexually competent tissues and at discrete locations hyphal knots are formed and later develop into female structures called protoperithecia. Special receptor hyphae called trichogynes emanate from the protoperithecia to search for male cells of the opposite mating type. Fertilization occurs by the fusion of the trychogyne and a male cell (i.e., plasmogamy). Following fertilization, the male- and the female-derived nuclei coexist in a heterokaryotic tissue and divide mitotically until they are sorted into dikaryotic tissue, in which each cell compartment

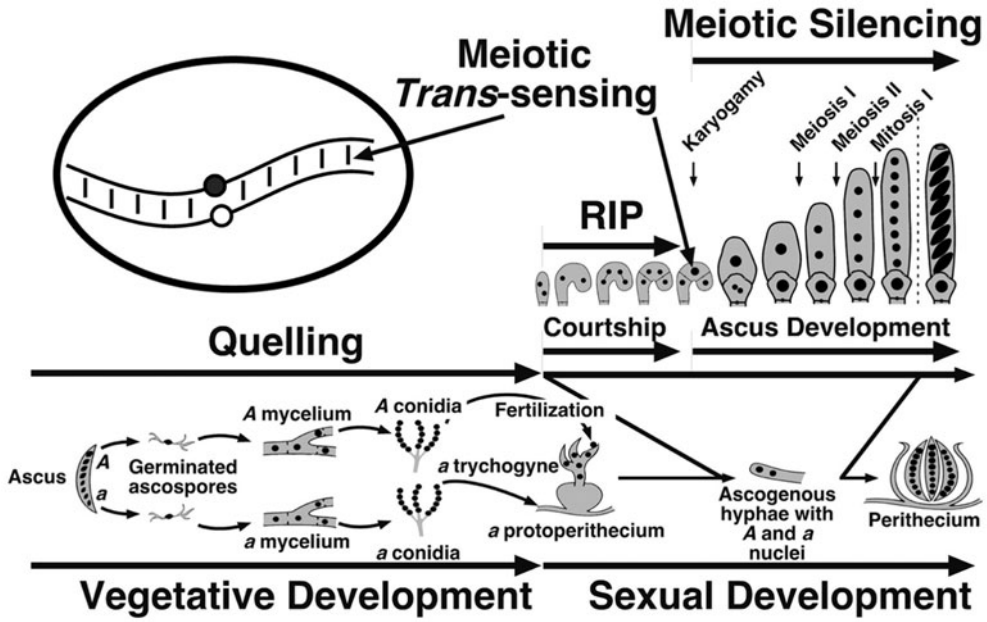


FIGURE 1 Genome defense mechanisms in the life cycle of *N. crassa*. *Neurospora* has two mating types, *A* and *a*. Sexual spores (ascospores) are formed when strains of opposite mating type mate and undergo sex. Germinated ascospores form mycelia from which asexual spores (conidia) are produced. Mating occurs when, in response to nitrogen starvation, a strain (either A or *a*) forms a protoperithecium (female element) that is fertilized by a male element of the opposite mating type to initiate perithecium development. After fertilization, the male- and the female-derived nuclei coexist in a heterokaryotic tissue and divide mitotically until they are sorted into a dikaryotic tissue (dikaryotic cells contain only one nucleus of each mating type). The nuclei then pair and undergo a series of synchronous mitoses until the tip of the hyphal cell in which they reside bends to form a hook-shaped cell called a crozier. One crozier will originate one ascus, containing eight ascospores. The time and places in the life cycle where quelling, RIP, meiotic *trans*-sensing, and meiotic silencing occur are indicated. Homologs pair inside the only diploid nucleus present in the zygote, immediately after karyogamy. The oval representing a diploid nucleus shows only one pair of chromosomes in the process of sensing. (Adapted from *Chromosome Research* **15:**633–651, 2007.)

contains only one nucleus of each mating type. The nuclei then pair and undergo a series of synchronous mitoses until the tip of the hyphal cell in which they reside bends to form a hook-shaped cell called a crozier. There, the two nuclei undergo a coordinated mitosis yielding, after septum formation, a uninucleate basal or antepenultimate cell, a uninucleate lateral or tip cell, and a dinucleate penultimate ascus mother cell that contains one nucleus of each mating type. It is in this ascus mother cell that karyogamy, meiosis, and postmeiotic mitosis take place. After the first mitosis, the resulting eight nuclei inside the ascus are then compartmentalized, resulting in an ascus cell that contains eight haploid spores arrayed in an order that reflects their lineage (Raju, 1980, 1992). One perithecium may contain up to 200 developing asci. Ascospores are then forcefully ejected from the beak of the perithecium. These ascospores, after ejection, continue to mature for a few days, after which they can be readily germinated following brief exposure to high temperature (~60°C for 40 min). Germinated ascospores produce vegetative mycelia, thus completing the sexual cycle. Because *Neurospora* is hermaphroditic in nature, strains of either mating type can act as either females or males. The ability to perform directional crosses adds to the already impressive power of *Neurospora* genetics. Meiotic segregation and recombination can be studied in *Neurospora* by analyzing individual asci (tetrads) or random spores ejected from numerous asci (Davis and de Serres, 1970; Perkins, 1966; Perkins, 1988).

GENOME DEFENSE IN *NEUROSPORA*

Genomes like that of *N. crassa* have developed a number of complex defenses to preserve their integrity (Borkovich et al., 2004; Galagan et al., 2003). At least four distinct but potentially interrelated mechanisms are known: DNA methylation, quelling, repeat-induced point mutation (RIP), and meiotic silencing by unpaired chromatin/DNA (Fig. 1). During haploid vegetative growth, AT-rich elements that invade the genome may be detected and densely methylated (Freitag et al., 2001; Miao et al., 2000; Romano and Macino, 1992; Singer et al., 1995). Multicopy elements can also activate quelling, a posttranscriptional silencing mechanism (Cogoni, 2001; Cogoni and Macino, 1999b; Cogoni and Macino, 2000; Pickford et al., 2002). Once activated, quelling produces diffusible signals (i.e., small interfering RNAs [siRNAs]) that block the propagation of the repeated element by RNAi-mediated degradation of its transcript (Catalanotto et al., 2002). If nuclei containing repeated DNA elements enter the sexual phase of the life cycle, the sequences are subjected to RIP (Freitag et al., 2002; Selker, 1990, 1997). In this process, GC-to-AT transition mutations are introduced into the duplicated sequences, presumably by unrepaired deamination of methylated cytosines. Many of the remaining, nonmutated cytosine bases are kept methylated by DIM-2, a DNA methyltransferase responsible for all known cytosine methylation in *Neurospora* (Kouzminova and Selker, 2001). Additionally, these sequences can be associated with trimethylated histone-H3, lysine-9 (H3K9me3) heterochromatin and transcriptionally repressed (Rountree and Selker, 1997; Tamaru et al., 2003). Remaining elements are scrutinized during meiosis by *trans*-sensing, which identifies the unsuccessful pairing of discrete DNA regions due to the presence of a heterologous region or the presence of a deletion on the opposite chromosome (i.e., the lack of a homologous region). This failure to "sense" a homologous region triggers an RNAi-mediated silencing mechanism that has been termed MSUD (meiotic silencing of unpaired DNA) (Shiu et al., 2001). Unfortunately, the acronym MSUD (maple syrup urine disease) is most commonly used to refer to a human disorder that abnormally affects the metabolism of amino acids and has been in use since 1975 (Wendel et al., 1975). To avoid further contamination of the scientific literature, we highly discourage its use.

Unlike quelling and RIP, there is no direct evidence that meiotic silencing is effective in preventing the spread of repeated elements in meiosis. The lack of active transposable elements in this organism and the fact that meiotic silencing can be detected in all laboratory strains tested as well as in many other isolates of this organism from nature suggest the possibility that meiotic silencing's true function/mechanism still remains to be discovered. One thing is clear, whatever that role might be, it must be related to some aspect of symmetry evaluation in meiosis. Based on what we know at this moment, however, the idea of meiotic silencing can be best explained in the context of a transposable infection (Fig. 2A). Imagine sex between two haploid parents (parents *A* and *a*), one of which was infected by a transposable element in the previous vegetative cycle. For simplicity, our discussion focuses on only one pair of homologous chromosomes (*Neurospora* has seven). These pairs of homologous chromosomes will first undergo premeiotic DNA replication, generating sister chromatids attached along their length by cohesin. The participating parental nuclei will then fuse at karyogamy, generating a diploid nucleus. Early in meiosis these homologous chromosomes will first *trans*-sense (i.e., evaluate their degree of mutual identity) and then synapse with each other, a process characterized by the distinct formation of a proteinaceous structure called the synaptonemal complex, which forms between the homologs (reviewed by Raju, 1980; and Zickler, 2006). In our model, the new transposable element, along with all of the other regions on the homologous chromosome, will undergo *trans*-sensing with the regions on the homolog. However, the transposable element will fail to detect a homologous region at the allelic location on its homolog and will be identified and tagged as unpaired DNA or chromatin. In *Neurospora* this would have a meaningful consequence, because the detection of this unpaired chromatin/DNA would trigger meiotic silencing. This would result in the production of a signal, perhaps siRNAs or Piwi-interacting RNAs (piRNAs), that would now target any transcript(s) from this unpaired region and from any other homologous region(s) of the genome for destruction. Once activated, meiotic silencing continues to occur as homologous chromosomes separate at the end of meiosis I, as sister chromatids separate at meiosis II and until after the first mitosis. It is at this stage, when each nucleus is enveloped in a membrane that signals the start of the process of cellularization, when the nuclei are encapsulated into individual ascospores, that meiotic silencing stops and silencing is reset.

By using these silencing mechanisms, *Neurospora* can protect its genome from parasitic nucleic acids during all stages of its life cycle. Perhaps providing evidence for the combined effectiveness of these strategies, there is only one known functional transposable element in *Neurospora*, and this is present in only 1 of more than 300 wild isolates of *Neurospora* from around the world (Kinsey, 1989; Kinsey and Helber, 1989). Attesting to the functionality of these mechanisms, when this transposable element was introduced into laboratory strains of *Neurospora*, its copy number was repressed in a quelling-dependent manner (Nolan et al., 2005) and existing duplications were inactivated by RIP (Cambareri et al., 1994). Similarly, it is clear that other transposable elements

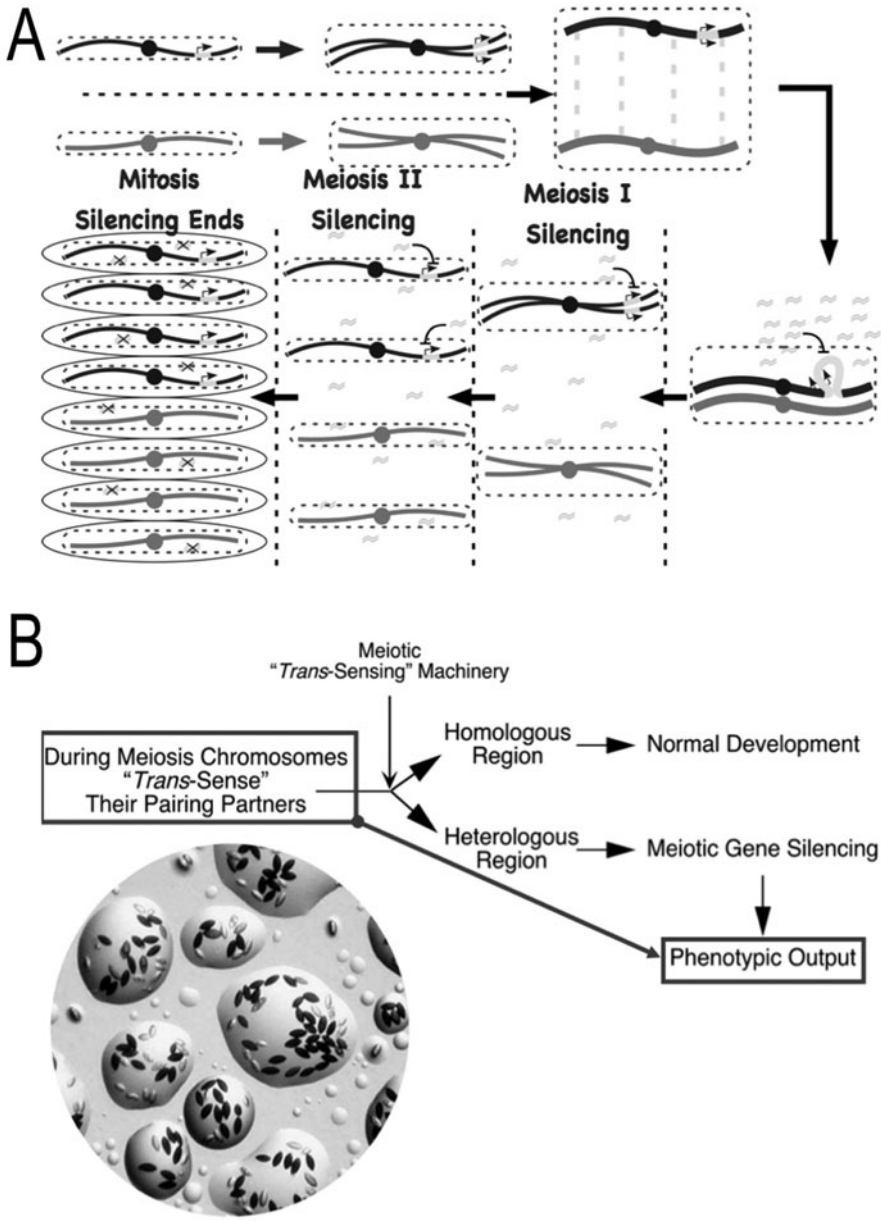


FIGURE 2 Meiosis, meiotic *trans*-sensing, and meiotic silencing. (A) Homologous chromosomes are represented by the squiggly lines contained inside the dashed rectangles. The temporal stage during which the prekaryogamic events occur inside the haploid A and *a* nuclei is represented by the horizontal dashed line. The stages where cell division occurs are indicated by vertical lines. The active comparison between chromosomal regions that occurs during meiotic *trans*-sensing and recombination is represented by the vertical dashed lines connecting the homologous chromosomes participating in the process. The clear region located on one chromosome represents a transposable or an insertion element. The small squiggly lines represent a hypothetical diffusible signal containing the sequence information from the unpaired region. Meiotic silencing uses this sequence information to silence homologous regions. (B) Depiction of the close connection seen in *Neurospora* between meiotic *trans*-sensing and silencing. The resultant phenotypic output, which may or may not reveal the result of meiotic gene silencing, can be detected by the use of appropriate reporters, which, according to their nature can affect the color and/or the shape of the ascospores product of the cross. The close temporal and developmental connection between meiosis and spore formation makes of *Neurospora* one of the most, if not the most powerful model system available for studying the molecular mechanisms behind meiotic *trans*-sensing. See the text for more details.

have tried their luck in *Neurospora* and failed, as evidenced by the presence of their RIP-mutagenized relics in the genome (Selker, 1990). Given this extensive "genome paranoia," it is not surprising that duplicated elements of any sort, including paralogous genes, are rare in *Neurospora* (Borkovich et al., 2004; Galagan et al., 2003).

THE DISCOVERY OF MEIOTIC SILENCING

The study of mutants affecting sexual development in *Neurospora* started in 1935 (Dodge, 1935, 1946). In 1972 and 1975 female and male sterile mutants, respectively, of *N. crassa* were identified (Mylyk and Threlkeld, 1974; Vigfusson and Weijer, 1972; Weijer and Vigfusson, 1972). These studies were then followed in 1975 by what is perhaps the earliest description of a *Neurospora* meiotic mutant, *meiotic-1* (*mei-1*), by David Smith (Smith, 1975). In 1978, Thomas Johnson published an elegant study on the isolation and characterization of perithecial development mutants in *N. crassa* (Johnson, 1978), a study that was followed by a report by A. M. DeLange in 1980, describing an exhaustive search for meiotic mutants (DeLange and Griffiths, 1980a, 1980b). In all these early studies, the key signature for meiotic mutants of *Neurospora* was, invariably, the production of white ascospores. These were interpreted as being the result of irregular segregation of chromosomes during meiosis. The rule was as follows: white or hypohaploid ascospores are aborted aneuploid products of meiosis, while reciprocal hyperhaploid ascospores develop normal black pigmentation and have good germination and viability (Smith, 1975). Concurrently with the search for meiotic mutants, the development of DNA-mediated transformation for *Neurospora* (Case et al., 1979) opened the way for the discovery of RIP (Selker, 1990). Hints of the instability of repeated sequences in *Neurospora* came from the strikingly different behavior observed for tandem duplications during the vegetative and the sexual phases of the life cycle. During vegetative growth, tandem duplications tend to be relatively stable. Duplications resulting from integration of transforming DNA by homologous recombination are lost at low but detectable frequency. In contrast, these same repeats are not stable in the sexual phase of the life cycle. Because the primary transformants were generally heterokaryotic (i.e., carried both transformed and untransformed nuclei), it was a common laboratory practice to obtain homokaryotic transformants by crosses prior to their characterization. Results from these crosses were not always clear. The transformation markers participating in these crosses were commonly observed to give a non-Mendelian inheritance, whose explanation was attributed to be the result of RIP mutagenesis of the transformation-generated repeats (Case, 1986; Case et al., 1979; Dhawale and Marzluf, 1985; Kim and Marzluf, 1988; Selker, 1990). The discovery of RIP opened a new and arguably very convenient way to inactivate genes in *Neurospora*, but unfortunately, it also obscured the identification and delayed the discovery of meiotic *trans*-sensing and silencing in *Neurospora*, because mutants in genes that might have been good targets for silencing could not have been obtained by crosses.

The discovery of meiotic *trans*-sensing and silencing was the result of the generation of an *Ascospore maturation-1* (*Asm-1*) deletion mutant of *Neurospora* by standard gene replacement and not by RIP (Aramayo and Metzenberg, 1996). We determined that the product of *asm-1*$^{+}$ (ASM-1) is an abundant nuclear protein essential for the formation of aerial hyphae, the development of protoperithecia, and the maturation of the ascospores (Aramayo and Metzenberg, 1996; Aramayo et al., 1996). Strains carrying deletion alleles of *Asm-1* do not form aerial hyphae but instead form "stunted" hyphae, a phenotype that resembles the one observed for the equivalent mutant of *Aspergillus nidulans* (Breakspear and Momany, 2007; Busby et al., 1996; Dutton et al., 1997; Miller et al., 1991, 1992; Wu and Miller, 1997). They are also female sterile, as revealed by their inability to form protoperithecia. A DNA fragment carrying both the promoter and coding region of the gene integrated at an ectopic location can readily complement these vegetative phenotypic defects. Obviously, the ectopically located complementing fragment can direct the production of wild-type transcripts and protein products and complement the deletion defect. These observations clearly established that *Asm-1*$^{\Delta}$ deletion alleles are recessive to the inserted complementing fragment, a property these alleles share with frameshift mutant alleles of the gene (e.g., *asm-1*^{fs}).

Despite the fact that both *Asm-1*$^{\Delta}$ deletion alleles and *asm-1*^{fs} frameshift alleles have the same vegetative mutant phenotype, these alleles have strikingly different genetic behavior in meiosis. During sexual development, the spores that inherit recessive, loss-of-function alleles of *Asm-1* cannot mature, whereas spores containing wild-type alleles mature normally. This was established by observing that in *asm-1*$^{+}$ × *asm-1*^{fs}, spores that carry the *asm-1*^{fs} allele do not mature, whereas those inheriting wild-type versions of the gene do. Given that the gene product of *Asm-1* is a protein that carries all the signatures of a DNA binding protein, we postulated that ASM-1 is a transcription factor required for the expression of genes involved in ascospore maturation. That is, its gene product is not required for meiosis I and II or mitosis I and II, but it is required to jump-start the system only after cellularization. This would explain the spore-specific requirement of the gene product.

Surprisingly, we observed that in *asm-1*$^{+}$ × *Asm-1*$^{\Delta}$ crosses, the *Asm-1*$^{\Delta}$ deletion allele was ascus dominant. That is, all spores within the ascus fail to develop, including the ones carrying the *asm-1*$^{+}$ allele. The behavior of *Asm-1*$^{\Delta}$ alleles was both puzzling and apparently contradictory, as the gene was being dominant by being absent. These observations were originally regarded to be the result of genetic "haploinsufficiency," a genetic term that describes a diploid phenomenon by which the product of a single functional allele of a gene is unable to confer a wild-type phenotype to the organism. Of course, if this were the case with *Asm-1*$^{\Delta}$ and the developmental deficiency observed were in fact due to haploinsufficiency, then a cross containing any two functional copies and the deletion would remedy the spore maturation defect. This could be tested by isolating a strain carrying the deletion allele and a functional copy of the gene inserted ectopically and then crossing it to a wild-type strain [i.e., *asm-1*$^{+}$ × *Asm-1*$^{+}$ (ectopic)]. However, even in this case a crop of immature spores was also observed. These results could not be easily reconciled with the idea that the dominance of *Asm-1*$^{\Delta}$ was due to haploinsufficiency (Aramayo and Metzenberg, 1996). Rather, they suggested that the unpaired copies of the gene were in fact misbehaving.

But how could genes be dominant by being absent in the homologous chromosome? The apparent genetic contradiction was resolved by the discovery of the connection between this phenomenon and RNA silencing (Shiu et al., 2001). Metzenberg and colleagues demonstrated that the meiotic silencing of unpaired DNA appears to be posttranscriptional because mutations in an RNA-dependent RNA polymerase (RdRP) gene, *Suppressor of ascus dominance-1* (*Sad-1*), suppress the ascus dominance exerted by unpaired copies of

Asm-1 or other reporter genes (Shiu et al., 2001). In light of this observation, in an *asm-1*$^{+}$ × *Asm-1*$^{\Delta}$ cross, what is really happening is that the unpaired wild-type allele is exerting its dominance by producing an abnormal diffusible product as a result of its failure to pair with its partner in the homologous chromosome. Further experiments demonstrated that pairing of the *Asm-1* gene, regardless of where it occurs, is essential for its normal expression and ascus development, and it was postulated that paired alleles could "sense" (i.e., *trans*-sense or undergo meiotic *trans*-vection) the presence or absence of their partners in homologous chromosomes (Aramayo and Metzenberg, 1996). Furthermore, failure to sense an intact and appositionally localized copy on the homologous chromosome caused silencing of copies positioned elsewhere in the genome, regardless of their own pairing status (Lee et al., 2004; Shiu et al., 2001). Meiotic *trans*-sensing and meiotic silencing are two highly interrelated but different mechanisms that together scan and control the integrity of the genomes that participate in meiosis.

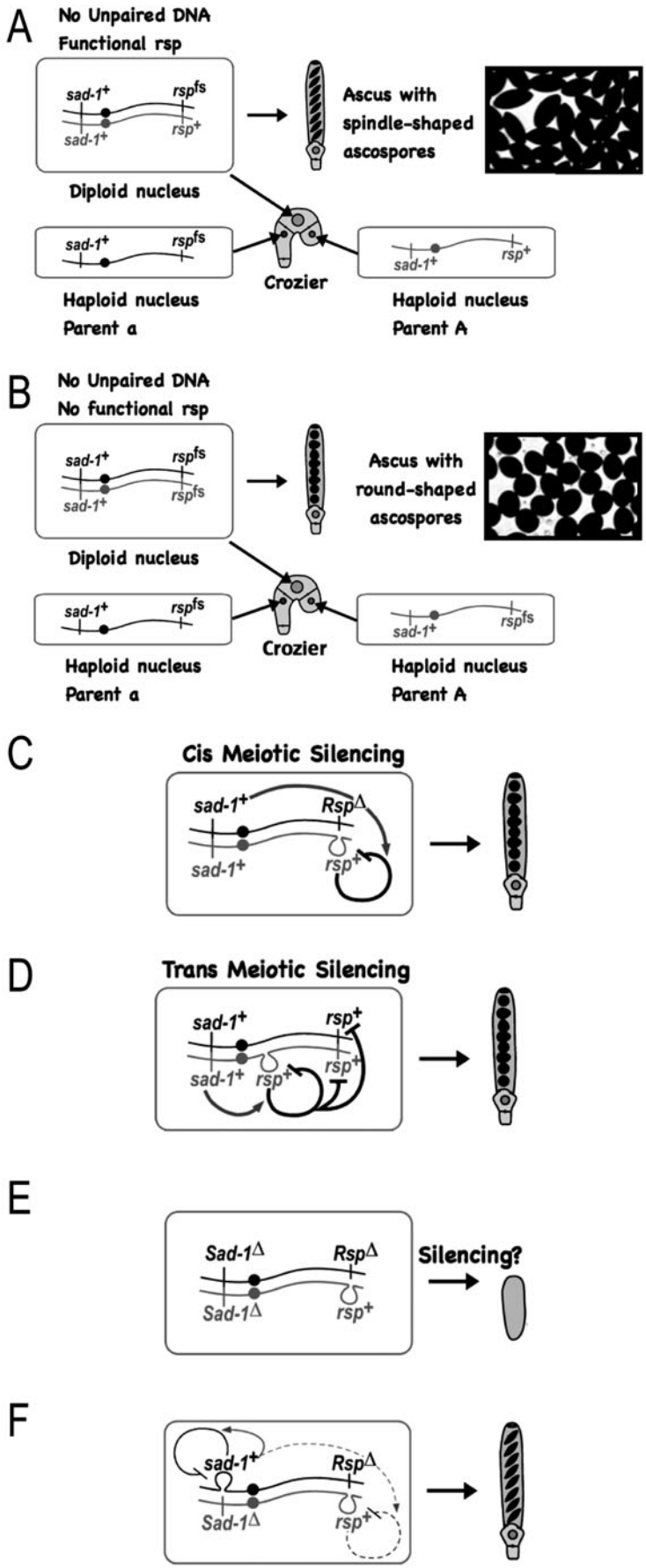
INDUCTION AND DETECTION OF MEIOTIC SILENCING

Chromosomes appear to "sense" the presence of homology and/or identify small regions of nonidentity within their homologs in early meiotic stages. If the regional identities are equivalent above a certain threshold, development proceeds normally. If not, meiotic *trans*-sensing machinery is activated and targets homologous sequences in the genome, regardless of their own pairing status (Fig. 2B). Some of the quantitative and qualitative aspects of the sensing threshold have been addressed experimentally, and at least two types of silencing (i.e., *cis-* and *trans-*) have been described (see below) (Lee et al., 2004). Quantitatively, the following observations have been made. (i) Given one small loop and one large loop of unpaired DNA, both carrying the same identical length of DNA homologous to a set of paired reporter genes, the large loop will silence more efficiently than the smaller one. (ii) Given two loops of identical size, but one carrying twice as much DNA homologous to a set of paired reporter genes, this more homologous loop will silence more efficiently than the one carrying the same amount of homologous DNA. (iii) The silencing signal produced by an unpaired loop is confined to the unpaired region and does not "spread" to neighboring regions (e.g., paired reporter genes can be located next to a region of unpaired DNA without being significantly perturbed). (iv) The canonical promoter of a gene need not be present in the loop of unpaired DNA for a gene to be silenced. (v) Meiotic silencing does not affect the ability of a promoter to direct transcription at a later developmental time (Kutil et al., 2003; Lee et al., 2003, 2004; Pratt et al., 2004).

In theory, to detect meiotic silencing, any gene whose gene product is required for the completion of meiosis, the reestablishment of mitosis, the cellularization, and/or maturation of the ascospores can be used. Similarly, genes whose gene products are essential structural components of the ascus would work. However, experimentally it is easier to assay meiotic silencing by performing crosses in which a reporter gene, typically a native gene required for ascospore morphology or color, is unpaired. Importantly, the use of genes whose sexually specific gene products have already completed their function before or at karyogamy is not recommended, as they would escape silencing, as would any gene products that either are not expressed or are dispensable for sexual development, unless, of course, they are fused to a fluorescent marker like green fluorescent protein (*gfp*$^{+}$).

The ratio of ascospores with mutant and wild-type phenotypes can be determined using reporters whose silencing results in an abnormal spore phenotype. An example with the reporter gene *Roundspore* (*Rsp*) and the meiotic silencing component *Sad-1* is shown in Fig. 3. In crosses between wild-type strains and strains carrying recessive loss-of-function alleles of *Rsp*, e.g., a single-nucleotide insertion creating a frameshift (*rsp*^{fs}), the *Rsp* alleles from both parents detect sufficient homology by *trans*-sensing so as not to trigger meiotic silencing (Fig. 3A). In this cross the "normal" expression of the only functional allele of *Rsp* (i.e., *rsp*$^{+}$) remains unaltered. The gene product produced by this allele produces enough mRNA and protein to confer a "wild-type" phenotype to the developing genotypically heterozygous diploid ascus cell. All of the ascospores have a spindle-shaped morphology. As expected, crosses homozygous for the recessive loss-of-function allele of *Rsp* (i.e., *rsp*^{fs}) produce asci containing eight round ascospores (Fig. 3B). No functional *Rsp* gene product is present in this situation. In contrast, if the *rsp*$^{+}$ alleles participating in this cross cannot sense their partners on the opposite homologous chromosome, for instance, when the mating strain carries deletion alleles of *Rsp* (i.e., *Rsp*$^{\Delta}$), all ascospores have a round or ovoid morphology. This situation triggers *cis*-meiotic silencing (Fig. 3C), a process that requires the participation of SAD-1 and that of other gene products involved in the process. As a result, silencing of *rsp*$^{+}$ is effective in the ascus. Therefore, unlike alleles carrying point mutations, deletion alleles of *Rsp are* ascus dominant and their behavior violates Mendelian law (at least at the level of phenotype), since all ascospores appear to be mutant, even those that contain functional *rsp*$^{+}$.

It should be noted that, similarly to deletion alleles, some RIP alleles (mutant alleles generated through RIP mutagenesis) exhibit dominant behavior in meiosis. Significant sequence deviation between homologous alleles, as opposed to deletions or insertions, is also sensed and can trigger repression. The degree of identity that is required to escape *trans*-sensing and avoid triggering meiotic silencing suggests that the *Neurospora* system has very stringent standards for defining homology (Pratt et al., 2004). This has been assessed by crossing strains carrying wild-type alleles of meiotic reporter genes with strains carrying varying degrees of mutation (generated by RIP) in the homologous locus. The mutated alleles, if sufficiently different, would confer a dominant phenotype by triggering meiotic silencing and repressing the wild-type gene. Amazingly, as little as 6% sequence divergence (or 94% identity) could trigger low detectable silencing, yet a slight increase of identity to 97% (or 3% divergence) prevented significant silencing (Pratt et al., 2004). Furthermore, the methylation status of the RIP-mutated alleles shifted the sequence identity threshold, such that methylated mutant alleles were detected as having worse identity to the wild-type allele than unmethylated ones (Pratt et al., 2004). This suggests that either the 5-methylcytosines contribute to heterology as a "fifth base" or their effects on chromatin structure (by recruitment of methylated DNA binding proteins, etc.) impact on homology recognition. These contributions are not mutually exclusive, and thus, both may contribute to identification and pairing. We therefore refer to deletion and RIP alleles as indels and homologous inducers of meiotic silencing, respectively. Similarly, if a wild-type strain is crossed to a partner containing an ectopic copy of *Rsp* (e.g., by directed insertion at the *his-3* locus), the ectopic allele will be unpaired and will trigger the silencing of all *Rsp* alleles


A
No Unpaired DNA
Functional rsp
sad-1+
rspfs
sad-1+
rsp+
Ascus with
spindle-shaped
ascospores
Diploid nucleus
sad-1+
rspfs
Crozier
sad-1+
rsp+
Haploid nucleus
Parent a
Haploid nucleus
Parent A
B
No Unpaired DNA
No functional rsp
sad-1+
rspfs
sad-1+
rspfs
Ascus with
round-shaped
ascospores
Diploid nucleus
sad-1+
rspfs
Crozier
sad-1+
rspfs
Haploid nucleus
Parent a
Haploid nucleus
Parent A
C
Cis Meiotic Silencing
sad-1+
RspΔ
sad-1+
rsp+
D
Trans Meiotic Silencing
sad-1+
rsp+
sad-1+
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E
Sad-1Δ
RspΔ
Sad-1Δ
rsp+
Silencing?
F
sad-1+
RspΔ
Sad-1Δ
rsp+

regardless of whether the other alleles are themselves paired or unpaired. Again round ascospores are produced (Fig. 3D). We refer to the type of silencing where paired regions must be silenced as *trans*-silencing.

In *Neurospora*, testing the involvement of genes in meiotic silencing is not straightforward. The diploid nature of the meiotic cell makes it difficult to identify recessive alleles in genes involved in the process. In addition, the meiotic lethality observed in crosses homozygous for loss-of-function alleles complicates the conceptually simple experiment of assaying meiotic silencing in the complete absence of the candidate gene's gene product (e.g., *Sad-1* [Fig. 3E]). The best known experiments that can be done are to assay the degree of meiotic silencing in crosses heterozygous for the candidate gene (e.g., *Sad-1* [Fig. 3F]). In this situation, the *cis*-silencing is expected to significantly decrease the amounts of transcript and protein below that which is expected from a single functional gene. According to this logic, if there is enough active SAD-1 protein in heterozygous crosses (probably synthesized before the *sad-1*$^+$ gene is silenced) to allow meiosis to proceed, but not enough to maintain fully functional meiotic silencing machinery, we should then be able to determine the involvement of *sad-1*$^+$ in meiotic silencing. The suppression of meiotic silencing or the "silencing the silencer" strategy has been widely used in the identification of several components of meiotic silencing. Once meiotic silencing is activated, the process is dominant. It should be noted, however, that not all loss-of-function alleles of meiotic silencing components are equally dominant, perhaps as a result of their involvement in different steps of the meiotic silencing pathway. Ultimately, the output of these experiments is, by convention, a percentage of wild-type ascospores. For reporter genes, the stronger the silencing, the lower the percentage of ascospores or asci with a wild-type phenotype. For suppressors, the stronger the suppression of silencing, the higher the percentage of ascospores or asci with a wild-type phenotype. It should always be kept in mind that these numbers represent the output of two events, meiotic *trans*-sensing and meiotic silencing, and the relative contribution of each to this percentage is still unclear. For example, one can imagine that once induced, meiotic silencing is always 100% effective within an ascus. If so, the output is a direct measure of the efficiency of meiotic *trans*-sensing or of the frequency at which unpaired DNA is detected. The converse is also potentially true; unpaired DNA is always detected but the efficiency of meiotic silencing varies. Additionally, of course, the output could be a combination of both efficiencies.

At what level is the known meiotic machinery involved at this level of homology sensing? Recent data suggest that the stable long-distance pairing (i.e., alignment), short-distance pairing (i.e., synapsis), recombination, and segregation steps of chromosome pairing in meiosis are all dispensable for meiotic silencing. Mutations in *spo-11*, *ski-8*, *Msh-4*, *mei-1*, *Mei-2*, and *mei-3*, which cause severe defects or eliminate synapsis and/or recombination, do not abrogate gene-specific silencing despite global asynapsis (R. J. Pratt and R. Aramayo, unpublished data). *spo-11* and likely *ski-8* mutants fail to stably align their chromosomes (Bowring et al., 2006; Tesse et al., 2003). This suggests that *trans*-sensing and the triggering of meiotic silencing may occur during an earlier, unstable chromosome alignment stage of meiosis upstream of mechanisms guiding synapsis. Very little is understood about these early events in chromosome recognition; but it is conceivable that the mechanisms involved in this *trans*-sensing use features of homology recognition mechanisms present in all organisms. Alternatively, *trans*-sensing could occur through a novel parallel independent pathway, perhaps through a mechanism similar to programmed DNA elimination in ciliates (discussed below).

RNAi AND MEIOTIC SILENCING

A role for RNAi has been strongly inferred by the identification of genes required for *Neurospora* meiotic silencing. RdRPs have been identified as essential components of RNAi in several organisms, and a separate RdRP, QDE-1, has been shown to be essential for quelling, the vegetative RNAi silencing process (Cogoni and Macino, 1999a). Further screens for meiotic silencing factors identified two other additional RNAi-related genes: one coding for an Argonaute-like protein, *Suppressor of meiotic silencing-2* (*Sms-2*) (Lee et al., 2003); and the other coding for a Dicer-like protein, *Suppressor of meiotic silencing-3* (*Sms-3*) (Alexander et al., 2007; D. W. Lee and R. Aramayo, unpublished data). Several other *Sms* mutants have also been identified (R. J. Pratt, D. W. Lee, and R. Aramayo, unpublished data). In addition, the posttranscriptional gene silencing nature of meiotic silencing was confirmed using transgene reporters. Only regions containing homology to the reporter transcript result in silencing when unpaired (Lee et al., 2004). Based on these observations, a model for RNAi's involvement in meiotic silencing was postulated (see Fig. 2 in Lee et al., 2003).

The *Sad-1*, *Sms-2*, and *Sms-3* orthologs in the fission yeast *Schizosaccharomyces pombe* (Rdp1, Ago1, and Dcr1, respectively) constitute the main RNAi pathway in this organism (Volpe et al., 2002). The RNAi pathway in *S. pombe* is essential for normal chromosome biology, including heterochromatin formation (e.g., histone H3K9 methylation) and normal centromere and telomere functions (Martienssen et al., 2005). In contrast, strains carrying loss-of-function mutations in any of the components of the meiotic silencing pathway (i.e., *Sad-1*, *Sms-2*, or *Sms-3*) of

FIGURE 3 Meiotic silencing in *Neurospora*: detection and suppression. Simplified views of the genetic composition of the participants in, and of the predicted phenotypic outputs of, several different crosses are presented using *Rsp* as a reporter gene. (A) In crosses where the diploid is *sad-1*$^+$/*sad-1*$^+$ *rsp*$^+$/*rsp*$^{\text{frameshift (fs)}}$, silencing of the reporter gene *Rsp* is not activated due to the lack of unpaired chromatin/DNA. As a result, the sole functional copy of *Rsp* can complement the heterozygous *Rsp* condition, which results in the production of wild-type spindle-shaped ascospores. This observation establishes that the product of *Rsp* acts before cellularization and that all spores, regardless of their individual genotypes, are spindle shaped. (B) As expected, in crosses where the diploid is *sad-1*$^+$/*sad-1*$^+$ *rsp*^{fs}/*rsp*^{fs}, all spores are round, which establishes that it is the product of *Rsp* that controls spore shape. (C to F) A box represents the diploid stage. Inside it, only one homologous chromosome pair is depicted with the *Sad-1* and the *Rsp* loci indicated. A predicted ascus product of the cross is shown to the right of the diploid cell. Arrows represent SAD-1 activity, and bars represent silencing. The thickness of these lines represents relative levels of silencing or activity. (C) Unpaired *rsp*$^+$ resulting in *cis*-silencing. (D) Unpaired ectopic *rsp*$^+$ resulting in *trans*-silencing. (E) In the absence of functional product of *Sad-1*, development stops at the pachytene stage of meiosis I. This makes the evaluation of the presence or absence of silencing by use of *Rsp* impossible. (F) The dominance of the *Rsp*$^{\Delta}$ allele can be suppressed in crosses heterozygous for *Sad-1*. See the text for details.

Neurospora do not have a discernible vegetative phenotype. In addition, whereas DNA methylation in *Neurospora* requires a histone H3K9-specific methyltransferase, this activity is independent of RNAi (Freitag et al., 2004), and there is no evidence that transcriptional repression accompanies meiotic silencing (Lee et al., 2004). However, all of the genes required for meiotic silencing in *Neurospora* are also required for fertility (Lee et al., 2003; Shiu et al., 2001; Lee and Aramayo, unpublished), indicating that components of the meiotic RNA silencing pathway are required for the completion of sexual development and/or meiosis and that these processes are intimately codependent.

A MODEL FOR MEIOTIC RNA SILENCING

Once identified, regions of nonhomology are predicted to produce some form of aberrant RNA, which is converted into double-stranded RNA (dsRNA) by the action of the RdRP SAD-1 (Fig. 4). The presence of dsRNA triggers the initiation step of the pathway, which could involve the

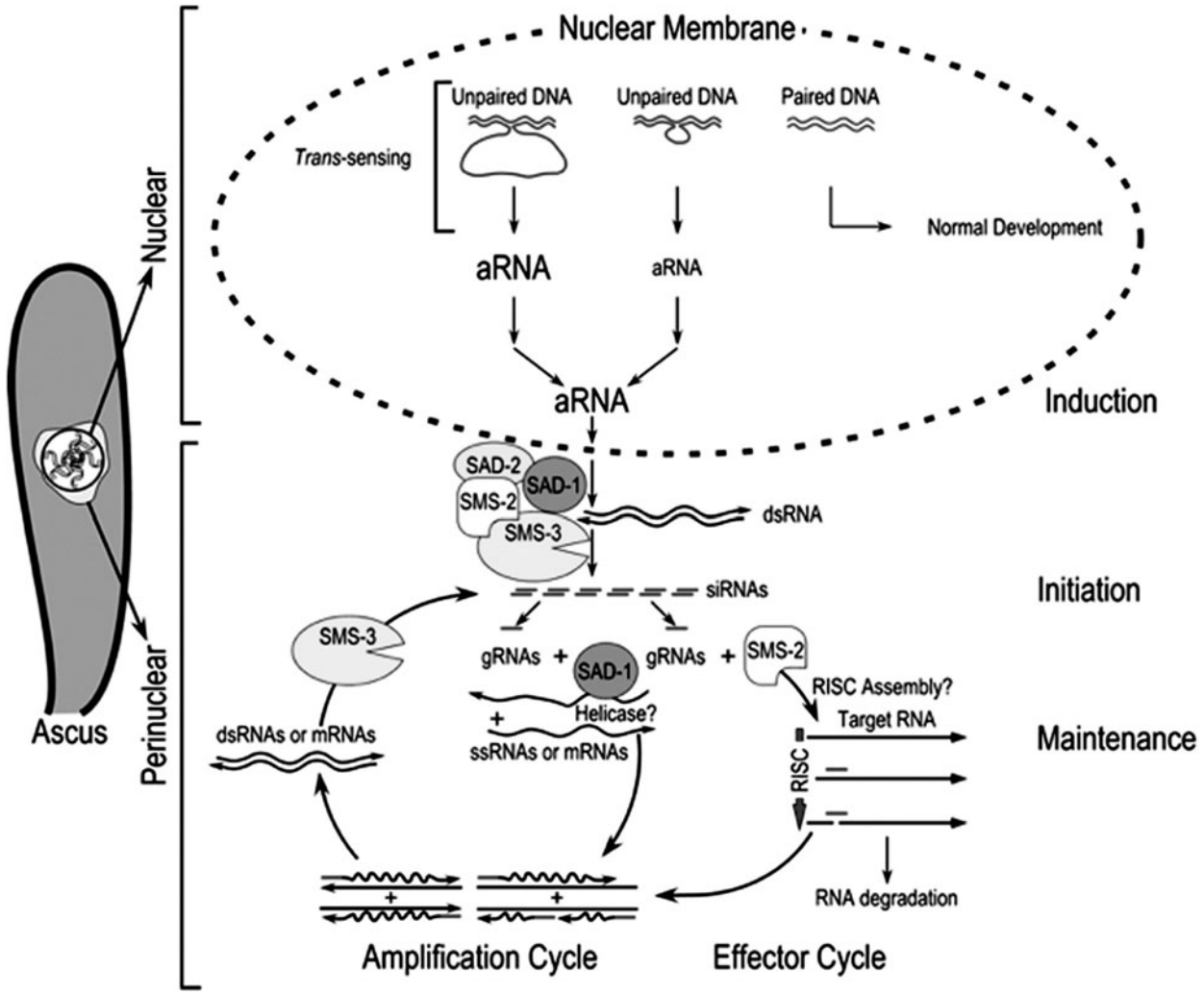

FIGURE 4 A model for meiotic silencing. An ascus at the pachytene stage of meiosis I is presented. Inside this cell, the meiotic nucleus, delineated by its nuclear membrane, apparently remains intact and is surrounded by a perinuclear structure that supports the attachment of components of the meiotic silencing apparatus. Inside the nucleus unpaired DNA (not paired DNA) induces meiotic silencing of homologous regions. The degree of unpairing determines the strength of the induction step, which presumably involves the synthesis of aberrant RNA (aRNA) and its conversion to double-stranded RNA (dsRNA) by the SAD-1 RdRP. The presence of dsRNA triggers the initiation of the meiotic RNA silencing process, which is composed of the following steps: the conversion of the dsRNA trigger into siRNAs via the SMS-3 Dicer (initiation step); the use of guide RNAs (gRNAs) as primers and single-stranded RNA (ssRNA) as a template by SAD-1 RdRP to generate dsRNA (amplification cycle); the incorporation of the gRNAs generated by both the initiation step and the amplification cycles into the RNA-inducing silencing complex (RISC), to direct the endonucleolytic cleavage of mRNA or ssRNA (effector cycle). It is possible that SAD-1 and SMS-2 maintain the silencing by participating in complexes related to the RdRP and the RNA-induced transcriptional silencing complexes detected in *S. pombe*, respectively.

conversion of the dsRNA trigger into siRNAs, although the presence of small RNAs with homology to the target sequence have not yet been observed. This step is predicted to be executed by the SMS-3/DCL-1 Dicer, because it is required for meiotic silencing (Borkovich et al., 2004; Catalanotto et al., 2004; Galagan et al., 2003; Lee et al., 2003). The maintenance of silencing probably involves amplification, also through SAD-1, and degradation of the target mRNAs via an RNA-induced silencing complex, of which the Argonaute-like protein SMS-2 is predicted to be an essential component, as in other conserved RNAi pathways (Lee et al., 2003). The lack of detectable siRNA-like molecules may be due to their rapid utilization and turnover, which may also contribute to the lack of silencing "memory" once sporulation is complete and homolog interactions are over (Lee and Aramayo, unpublished). Interestingly, the SAD-2 protein is also required for meiotic silencing, but its role appears to be to anchor or recruit the SAD-1 RdRP to the cytoplasmic face of the nuclear periphery (Shiu et al., 2006). This indicates that SAD-1's RdRP activity is required outside the nucleus, perhaps to screen RNAs as they transit nuclear pores as a sort of quality check. In any case, RdRP activity is likely a downstream effector of meiotic silencing and not a component of the *trans*-sensing mechanism that must necessarily be nuclear. An "RNA quality" monitoring system, however, implies that deficient pairing triggers production of aberrant transcripts to alert the system and hence that proficient recognition and pairing suppresses this transcription. Determining any role(s) for aberrant transcription in meiotic silencing awaits further analyses.

MEIOTIC SILENCING, A COMMON THEME IN MEIOSIS?

In the meioses of *Neurospora*, worms, and mice, gene expression from DNA that lacks a pairing partner is silenced. This meiotic silencing by unpaired DNA was first observed in *N. crassa* when it was shown that expression of a gene required for the maturation of sexual spores required proper meiotic pairing (Aramayo et al., 1996). These observations were reminiscent of the *trans*-vection phenomena previously observed in *Drosophila* (Lewis, 1954). Further investigations suggested that, rather than gene expression being activated or repressed by pairing, gene expression was being prevented by unpairing, probably by triggering an RNAi-like silencing mechanism acting on homologous mRNAs (Lee et al., 2003, 2004; Shiu et al., 2001).

Since then, meiotic silencing by unpaired chromatin/DNA has been discovered in both worms and mouse. Here, unpaired chromatin/DNA is associated with specific histone modifications and transcriptional silencing (Baarends et al., 2005; Bean et al., 2004; Turner et al., 2005). In worms, unpaired chromatin/DNA is imprinted and regains transcriptional activation at a slower rate than paired regions due to its ability to retain chromatin signatures related to silencing longer in the zygote (Bean et al., 2004). In mouse, meiotic silencing might be related to the meiotic sex chromosome inactivation observed during male spermatogenesis (Handel, 2004). The role of this inactivation is controversial but has been proposed as a mechanism for imprinting the paternal X chromosome for preferential inactivation in the murine placental tissues and marsupial embryos (Huynh and Lee, 2005; Okamoto et al., 2005). In both mouse and worms, there is a strong correlation between homologous and nonhomologous synapsis and transcriptional repression (Bean et al., 2004; Turner et al., 2005, 2006). In addition to the silencing of these large chromosomal regions, smaller unpaired regions in mouse spermatogenesis also seem to invite heritable epigenetic modifications and may be mechanistically related to the meiotic silencing phenomena observed in *Neurospora* (Herman et al., 2003; Rassoulzadegan et al., 2002, 2006).

While there are differences in the known properties of these phenomena, there is one key underlying theme: they all seem to identify regions of unpaired chromatin/DNA and target them for silencing. These silencing phenomena are considered epigenetic, because they result in a heritable, yet reversible, mutant phenotype in progeny that contain genes with a wild-type DNA sequence. By studying meiotic silencing in the genetically amenable and fast-growing filamentous fungus *N. crassa*, we expect to shed light on the properties and mechanisms of these and other potentially related epigenetic phenomena.

EVOLUTIONARY CONSERVATION AND FUNCTION OF MEIOTIC SILENCING

The ancient origins of meiotic silencing in all of its current manifestations are likely grounded in RNAi-mediated genome defense mechanisms. The role of RNAi in genome defense has been identified in many organisms. It is likely that the recognition of linear sequence heterology in meiosis, crucial for productive recombination, is also (and perhaps originally) a component of genome defense. The meiotic processes that we observe today may be partly a combined product of escalating warfare between genomes and invasive elements, with homolog recognition originating as a friend-or-foe assessment. Such *trans*-sensing and its connection to the meiotic recombination machinery are very poorly understood; but this *trans*-sensing could be related either to RNAi processes or to a separate process that has adapted RNAi mechanisms as the effector process in some organisms.

Although meiotic silencing is evident in highly diverse organisms, it appears to be absent in many as well, including yeast, flies, and at least some plants. Fission yeast, for example, seems to have all of the RNAi components required, albeit a minimal set, and has no meiotic silencing (A. Klar and R. Aramayo, unpublished data). However, recombination in this organism occurs without synapsis. Conversely, budding yeasts have synapsis but no RNAi components and no meiotic silencing (Anantharaman et al., 2002; Aravind et al., 2000). In *Drosophila,* synapsis is absent only in males, but there is no evidence for meiotic silencing in either sex, and an RdRP seems to be missing from the fly RNAi toolkit. Similarly, there are no obvious RNAi-related RdRPs encoded in mammalian genomes, yet mammals have both synapsis and meiotic silencing. However, meiotic silencing in mammals, as defined by meiotic sex chromosome inactivation and MSUC (meiotic silencing by unpaired chromatin), appears to be largely an adaptation of DNA repair pathways and, apparently, mechanistically quite distinct from RNAi. It remains to be determined, however, if the widespread antisense transcription observed in the mammalian transcriptome replaces the roles played by RdRPs in other organisms (Katayama et al., 2005). Finally, there are flowering plants, which have multiple isoforms of all RNAi components and full synaptonemal complex formation—but no clear demonstration as yet for meiotic silencing (V. Chandler, personal communication). A related phenomenon in plants, paramutation, is an interallelic, *trans*-sensing epigenetic process that requires an RdRP, *mop-1* (Chandler et al., 2000; Chandler and Stam,

2004). Paramutation may have originated from meiotic silencing but adapted later to operate in mitotic tissues.

The absence of either an RdRP or a synaptonemal complex may select against meiotic RNAi silencing or select for adaptation of a non-RNAi related mechanism. As mentioned for flies, the loss of meiotic synapsis could predispose for a further loss of meiotic silencing, if unsynapsed chromatin can be targeted for repression by this process. Similarly, loss of synapsis in fission yeast could have caused the loss of a mechanistic link between RNAi and meiotic silencing. The loss of an RdRP, on the other hand, would not cripple RNAi-related mechanisms but might decrease their sensitivity to endogenous substrates such as unpaired DNA.

Irrespective of the effector response in meiotic silencing, all sexually reproducing organisms must utilize some aspects of *trans*-sensing to detect homology between chromosomes while also creating a barrier to heterologous pairing. A number of transvective processes have been identified in diverse species—operating either within or outside meiosis. In many cases, these processes either have been determined or are predicted to have a mechanistic link to RNAi (Alleman et al., 2006; Herman et al., 2003; Rassoulzadegan et al., 2006; Wu and Morris, 1999). Genomic DNA elimination in *Tetrahymena* is a dramatic example of a *trans*-sensing process that involves RNAi (for a more informative review of this complex process, see Mochizuki and Gorovsky, 2004). These ciliates have two nuclei that share one cytoplasm: a transcriptionally inactive, diploid micronucleus and a transcriptionally active, polyploid macronucleus. The macronuclear DNA is a fragmented and amplified version of the micronucleus in which all repetitive DNA elements, including transposons, have been deleted (reviewed by Matzke and Birchler, 2005; and Mochizuki and Gorovsky, 2004). After conjugation, meiosis, and genome mixing, the old macronucleus is destroyed and a new macronucleus arises from a new, genetically mixed micronucleus—but any sequence not present in the old macronucleus is eliminated during new macronuclear maturation. The recognition process—a *trans*-sensing process between two separate nuclei—involves production of small, scanning RNAs from micronuclear transcription that are proposed to "scan" the old macronuclear genome. Those with homology (e.g., encoding essential loci) are degraded, whereas those with no homology (e.g., repetitive elements) are then used to guide elimination of homologous sequences in the new macronucleus. This results in a fragmented genome in which all repetitive elements have been eliminated, and the remaining gene-encoding sequences are amplified and transcribed during vegetative growth. The elimination-targeting mechanism is highly related to RNAi-mediated transcriptional silencing in plants and *S. pombe*, but ciliates have clearly found a more permanent way to silence the targets—by deleting them. It is interesting to consider that related genome-scanning mechanisms may be at work in meiotic *trans*-sensing.

From a genome protection perspective, it makes sense that organisms have developed a molecular mechanism like meiotic silencing to check the integrity of the genomes participating in meiosis. Meiotic silencing allows prevention of the expression of novel insertions (e.g., transposons) without necessarily affecting the regulation of adjacent genes. From an evolutionary perspective, however, meiotic silencing could also participate in speciation. Genomes evolving significant differences in genes required for germ cell/spore viability or meiotic progression could trigger *trans*-sensing alerts and meiotic silencing, leading to reproductive isolation from each other. In this context, Shiu et al. (2001) showed that mutations in *Sad-1*-RdRP significantly increased interbreeding efficiencies among various *Neurospora* species, indicating that a component of the species barrier in this organism is meiotic silencing (Shiu et al., 2001). Genomes could also accumulate a series of mutations or duplications in regions that house genes that are not required for meiosis without consequences to fertility. Heterochromatic regions of genomes, for example, may have become transposons "traps" partly because they are regions which transposons can invade without creating a meiotic silencing consequence. Heterozygous (novel) insertions would be recognized by *trans*-sensing and repressed to prevent further spread, but unless the genomic neighborhood housed meiotic loci that would be affected by any potential spreading of the repression, there would be no consequence to fertility. Duplications of genes involved in development, considered a major source of evolutionary complexity, would also be allowed.

The study of meiotic *trans*-sensing and meiotic silencing is therefore important not only from a mechanistic perspective (i.e., to understand how chromosomes pair and how homologous regions are detected) but also from an evolutionary point of view. Meiotic silencing may be another reproductive fitness test in the assessment of genomes that are brought together by sexual reproduction, and its potential role in genome evolution merits consideration.

We apologize for the exclusion, due to limited-space considerations, of information pertinent to organisms other than those included here. We also thank our colleagues and members of the Aramayo Lab for helpful discussions and/or the sharing of data unpublished at the time of this writing: Dong Whan Lee, Ana Victoria Suescun, Alexis Brown, Ryan Millimaki, and Aldrin Lugena. This work was supported by U.S. Public Health Service Grants GM58770 to R.A.

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12

Mycoviruses

DONALD L. NUSS

Viruses of fungi (mycoviruses) were discovered much more recently than viruses of plants and animals and, with few exceptions, remain less well studied. This is understandable when one considers the modest economic importance of mycovirus infections, the relatively small size of the fungal research community, and the fact that the majority of mycovirus infections are asymptomatic. However, the last several decades have witnessed an increased interest in mycoviruses and a significant acceleration in the pace of mycovirus research. This has been driven, in part, by increased reports of mycovirus-mediated phenotypic changes in their fungal hosts that are considered to be fundamentally interesting and of potential practical value. Interest has been further stimulated by technical advancements in mycovirus molecular biology that have demonstrated the utility of mycoviruses as tools for probing mechanisms underlying fungal biology and for manipulating the fungal host. Complementary advances in the genomics of filamentous fungi are providing experimental systems with the rare capacity for facile genetic manipulation of both virus and eukaryotic host. This chapter provides a brief overview of viruses associated with filamentous fungi while highlighting recent developments in mycovirus molecular biology that illustrate the potential utility of mycoviruses for fundamental research and practical applications.

BRIEF OVERVIEW OF MYCOVIRUSES OF FILAMENTOUS FUNGI

Viruses of filamentous fungi, with the exception of the double-stranded DNA (dsDNA) rhizidioviruses isolated from the aquatic fungus *Rhizidiomyces* (Dawe and Kuhn, 1983), have genomes composed of RNA and are accommodated primarily within six taxonomic families: *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Reoviridae*, *Narnaviridae*, and *Hypoviridae* (Table 1). Members of the family *Totiviridae* form icosahedral virions of approximately 40 nM in diameter that contain a single 4.6- to 7-kbp dsRNA (Wickner et al., 2005). The single genomic RNA encodes a capsid protein and an RNA polymerase protein that consists of the capsid protein fused with an RNA-dependent RNA polymerase (RDRP) domain as a result of a −1 ribosomal frameshift. Viruses of related genera within the family are found in the protozoan hosts *Giardia*, *Trichomonas*, and *Leishmania*.

Members of the family *Partitiviridae* also form small icosahedral virions, but the genome consists of two dsRNAs (1.4 to 2.2 kbp) that are individually packaged (Ghabrial et al., 2005a). One dsRNA encodes the capsid protein, and the second dsRNA encodes the RDRP. Viruses of two related genera of the *Partitiviridae* infect plants.

The family *Chrysoviridae* was originally listed as a genus of the family *Partitiviridae*. Members of this family also form 30- to 35-nm virion particles, but the genome consists of four unrelated, separately encapsidated dsRNAs of 2.4 to 3.6 kbp in size (Ghabrial et al., 2005b). Similar to what occurs for members of the *Partitiviridae*, the capsid protein and RDRP of members of the *Chrysoviridae* are encoded on separate dsRNAs. The functions of the proteins encoded by the other two dsRNAs have not been determined.

Reoviruses that infect fungi are organized within the genus *Mycoreovirus* of the extensive family *Reoviridae* (Mertens et al., 2005). Members of this genus contain 11 or 12 segments of dsRNA (732 to 4,127 bp) that are packaged within a large, ~80-nm, double-shelled spherical virus particle. Hosts for viruses constituting the 12 genera of the family *Reoviridae* include plants, animals, invertebrates, and lower eukaryotes.

Members of the families *Narnaviridae* and *Hypoviridae* are found only in fungal hosts and are distinguished by the absence of a capsid protein and, consequently, the inability to form true virions. The family *Narnaviridae* is comprised of the genus *Narnavirus*, which is restricted to viruses that infect *Saccharomyces cerevisiae*, and the genus *Mitovirus*, which contains viruses of filamentous fungi (Buck et al., 2005). The mitovirus genome consists of a 2.3- to 2.7-kb single-stranded RNA (ssRNA) that encodes a single protein containing RDRP motifs. Mitoviruses are associated with mitochondria, and the coding strand employs mitochondrial codon usage.

Confirmed members of the family *Hypoviridae* infect only the chestnut blight fungus *Cryphonectria parasitica* and are related phylogenetically to the potyviruses, a large group of

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TABLE 1 Primary mycovirus taxonomic families[a]

Family	Nature of genome	Genome size	Genome configuration	Virion[b]	Morphology (size in nm)
Totiviridae	dsRNA	4.6–7 kbp	1 segment	+	Icosahedral (40)
Partitiviridae	dsRNA	1.4–2.2 kbp	2 segments	+	Icosahedral (30–35)
Chrysoviridae	dsRNA	2.4–3.6 kbp	4 segments	+	Icosahedral (30–35)
Reoviridae	dsRNA	0.7–4.1 kbp	11 or 12 segments	+	Isometric (80)
Narnaviridae	ssRNA	2.3–2.7 kb	1 segment	−	Ribonucleoprotein complex
Hypoviridae	ssRNA	9–13 kb	1 segment	−	Pleomorphic membrane vesicles

[a]The unassigned genus *Rhizidovirus* contains dsDNA viruses from the aquatic fungus *Rhizidiomyces* (Dawe and Kuhn, 1983), and the genus *Barnavirus* contains the ssRNA, 4-kb, 1-segment, bacilliform virus particle found in the cultivated mushroom *A. bisporus* (Wright and Revill, 2005).

[b]+, virions are formed; −, no virions are formed.

ssRNA plant viruses (Nuss et al., 2005). Hypoviruses were originally classified as dsRNA viruses due to the prominence of dsRNA found in extracts of infected *C. parasitica*. Subsequent cloning, sequence analysis, and development of an infectious hypovirus cDNA clone demonstrated that the hypovirus coding strand is infectious, a defining characteristic of ssRNA viruses (Shapira et al., 1991; Choi and Nuss, 1992). Hypovirus genetic information consists of a single linear RNA of 9 to 12.7 kb that encodes one polyprotein (species CHV3 and CHV4) or two polyproteins (species CHV1 and CHV2) (Hillman and Suzuki, 2004). Hypovirus RNA replication is associated with pleiomorphic membrane vesicles of 50 to 80 nm in diameter. While the six taxonomic families described above accommodate the majority of mycoviruses, a growing list of recently characterized mycoviruses remains unclassified (Table 2) (Marquez et al., 2007; Kwon et al., 2007). Additional adjustments to the current taxonomic structure will be required as more mycovirus sequences become available.

Mycovirus infections share several features that are distinctive from plant and animal viruses. Perhaps the most significant difference is that the life cycle of mycoviruses is not punctuated by an extracellular transmission phase. Mycoviruses are not infectious in the classic sense. Mycovirus infections cannot be initiated by exposing uninfected hyphae to cell extracts prepared from an infected strain. Rather, mycoviruses are transmitted by cytoplasmic mixing following hyphal fusion (anastomosis) or through asexual spores. Mycovirus infections are also persistent. In this regard, the absence of capsid proteins for members of two mycovirus taxonomic families, *Hypoviridae* and *Narnaviridae*, is likely related to the persistent nature and intracellular confinement of mycovirus life cycles. Most mycoviruses lead secret lives, causing no obvious symptoms in their fungal hosts.

TABLE 2 Examples of mycovirus-mediated alteration in fungal host phenotype[a]

Symptom	Fungus	Plant host	Disease	Mycovirus family	Reference
Thermal tolerance	*C. protuberata*	*D. lanuginosum* and tomato	NA	Unclassified	Marquez et al., 2007
Reduced mycotoxin production	*F. graminearum*	Wheat	Scab disease of small grain	Unclassified; potexvirus-like genome	Kwon et al., 2007
Hypovirulence	*Diaporthe perjuncta*	Stone fruits	Diaporthe disease of stone fruit	Unclassified; tombusvirus-like	Moleleki et al., 2003
Hypovirulence	*Ophiostoma ulmi*, *Ophiostom a novo-ulmi*	Elm trees	Dutch elm disease	*Narnaviridae* (genus *Mitovirus*)	Buck and Brasir, 2002.
Hypovirulence	*Sclerotinia homoeocarpa*	Turfgrass	Dollar spot disease	*Narnaviridae* (genus *Mitovirus*)	Deng et al., 2003
Hypovirulence	*Rhizoctonia solani*	Potato	Rhizotonia disease	Unclassified	Lakshman et al., 1998
Hypovirulence	*Helminthosporium victoriae*	Oats	Victoria blight	*Totiviridae* and *Chrysoviridae* coinfection	Jiang and Ghabrial, 2004
Hypovirulence	*Rosellina necatrix*	Apple	White root rot	*Reoviridae*	Wei et al., 2003
Hypovirulence	*H. mompa*	Fruit trees	Violet root rot	*Endornavirus*	Osaki et al., 2006
Hypovirulence	*C. parasitica*	American chestnut	Chestnut blight	*Hypoviridae*, *Reoviridae*, *Narnaviridae* (genus *Mitovirus*)	Hillman and Suzuki, 2004

[a]Only mycoviruses for which the nucleotide sequence has been determined are listed. NA, not applicable.

However, exceptions to this general feature, discussed in the next section, include host phenotypic changes that are fundamentally interesting and of potential practical value.

SYMPTOMS ASSOCIATED WITH MYCOVIRUS INFECTIONS

Efforts to understand the cause of symptoms associated with severe dieback disease, La France disease, of the cultivated mushroom *Agaricus bisporus* led to the discovery of the first mycovirus in 1962 (Hollings, 1962). Symptoms associated with mycovirus infections remain a significant economic consideration to the cultivated mushroom industry due to the cost of hygienic precautions implemented to prevent virus epidemics and the emergence of new virus diseases such as MX virus (Rao et al., 2007). Interestingly, the majority of the symptoms associated with mycovirus infections have been viewed as having potential utility and quite often influence interactions between the fungal host and other organisms (Table 2). For example, the ability of the endophytic fungus *Curvularia protuberata* to confer heat tolerance to the panic grass *Dichanthelium lanuginosum* collected from geothermal soils in Yellowstone National Park was shown to require the presence of a mycovirus, subsequently named the Curvularia thermal tolerance virus (Marquez et al., 2007). Moreover, the virus-infected endophytic fungus, but not the virus-free fungus, was able to confer heat tolerance to tomato.

Most reports of mycovirus-associated symptom expression involve an alteration in the interaction between plant pathogenic fungi and their plant host. These infections generally result in reduced fungal virulence, termed hypovirulence, although cases of increased virulence have been reported (Nuss and Koltin, 1990). The best-characterized examples of hypovirulence (Table 2) primarily involve fungal members of the Ascomycota, and two basidiomycetes, *Rhizoctonia solani* and *Helicobasidium mompa*. The major taxonomic classes of mycoviruses are represented among the viruses that are associated with the hypovirulence phenotype, which also includes a number of interesting unclassified mycoviruses. Hypovirulence is often accompanied by additional symptoms. For example, the reduced-virulence phenotype exhibited by the FgV-DK21 virus-infected scab disease pathogen of small grains, *Fusarium graminearum*, is accompanied by reduced mycelium growth, increase pigment production, and, most interestingly, a significant reduction in the production of trichothecene mycotoxins (Kwon et al., 2007). Hypovirulence has not yet been reported for fungal pathogens of animals or humans.

The properties of mycovirus infections present technical challenges in demonstrating cause and effect relationships between mycovirus infection and symptom expression. It is often difficult to cure a fungal strain of a mycovirus infection. The reintroduction of a mycovirus into a cured strain is hampered by the absence of an extracellular phase in the mycovirus life cycle. The use of anastomosis to reintroduce mycovirus into a cured strain is complicated by the transmission of a mixture of cytoplasmic material. These technical problems have been overcome for one group of mycoviruses, the hypoviruses responsible for hypovirulence of the chestnut blight fungus *C. parasitica*. Not only did the development of an infectious full-length hypovirus cDNA clone allow the conclusive demonstration that the hypovirus genetic information was responsible for hypovirulence and associated symptoms, but it also provided the means for addressing the mechanisms underlying mycovirus-mediated alterations of fungal host phenotype and gene expression.

THE HYPOVIRUS-*C. PARASITICA* EXPERIMENTAL SYSTEM

The primary elements of the hypovirus-*C. parasitica* experimental system consist of full-length hypovirus cDNAs and a genetically manipulatable fungal host (Fig. 1). Hypovirus infections of *C. parasitica* can be initiated by installing the hypovirus cDNA, under the control of a fungal gene promoter and terminator flanking elements, into the host chromosome (transformation) (Choi and Nuss, 1992). The hypovirus cDNA-derived transcripts produced in the resulting "transgenic hypovirulent" strains are transported to the cytoplasm, translated, and used as a template to initiate hypovirus RNA replication. Replication occurs in association with cytoplasmic vesicles that can be purified from the fungus for in vitro analysis of viral RNA synthesis (Fahima et al., 1993). This process results in a replicating hypovirus RNA trimmed of all nonviral vector nucleotides (Chen et al., 1994a). Alternatively, infection can be initiated by electroporation-mediated introduction of the coding strand transcript generated in vitro from the viral cDNA (transfection) (Chen et al., 1994b). Both protocols require the production of cell wall-free spheroplasts from virus-free *C. parasitica*. The transformation protocol employs a selectable marker to allow isolation of resulting transgenic hypovirulent strains. After transfection, the regenerated hyphal structures anastomose, forming cytoplasmic networks through which the replicating hypovirus RNA can migrate. This allows efficient recovery of transfection-initiated hypovirulent strains without reliance on marker selection.

Progress in developing the hypovirus reverse genetics system has been accompanied by advances in *C. parasitica* molecular biology and genomics. DNA-mediated transformation of *C. parasitica* spheroplasts is very efficient, yielding several thousand stable transformants per microgram of transforming DNA (Churchill et al., 1990). Since *C. parasitica* is haploid and asexual spores are uninucleate, gene disruption and elimination of heterokaryons are quite efficient. The efficiency for homologous recombination has recently been increased from ~5 to 85% with the development of a *C. parasitica* mutant strain containing a disruption of the KU80 gene that encodes a key component of the nonhomologous-end-joining DNA repair pathway (Lan et al., 2008). The *C. parasitica* genome was recently sequenced by the U.S. Department of Energy Joint Genome Institute Community Sequencing Program (http://www.jgi.doe.gov/CSP/index.html). Finally, *C. parasitica* has been shown to support the replication of members of five virus families: *Hypoviridae*, *Reoviridae*, *Narnaviridae*, *Partitiviridae*, and *Chrysoviridae* (reviewed by Hilman and Suzuki, 2004), thus providing a rich resource for approaching fundamental issues of virus-host interactions.

MECHANISMS UNDERLYING MYCOVIRUS-MEDIATED MODULATION OF FUNGAL HOST PHENOTYPE AND GENE EXPRESSION

There has been a growing appreciation in contemporary virology research for the connection between virus-mediated symptom expression and alterations in cellular regulatory processes. In this regard, efforts to understand hypovirus-mediated symptom expression revealed alterations in several cellular signal transduction pathways and, in the process, provided the first link between G-protein signaling and fungal virulence.

The suggestion that hypovirus infection may alter one or more cellular regulatory pathways is derived from the

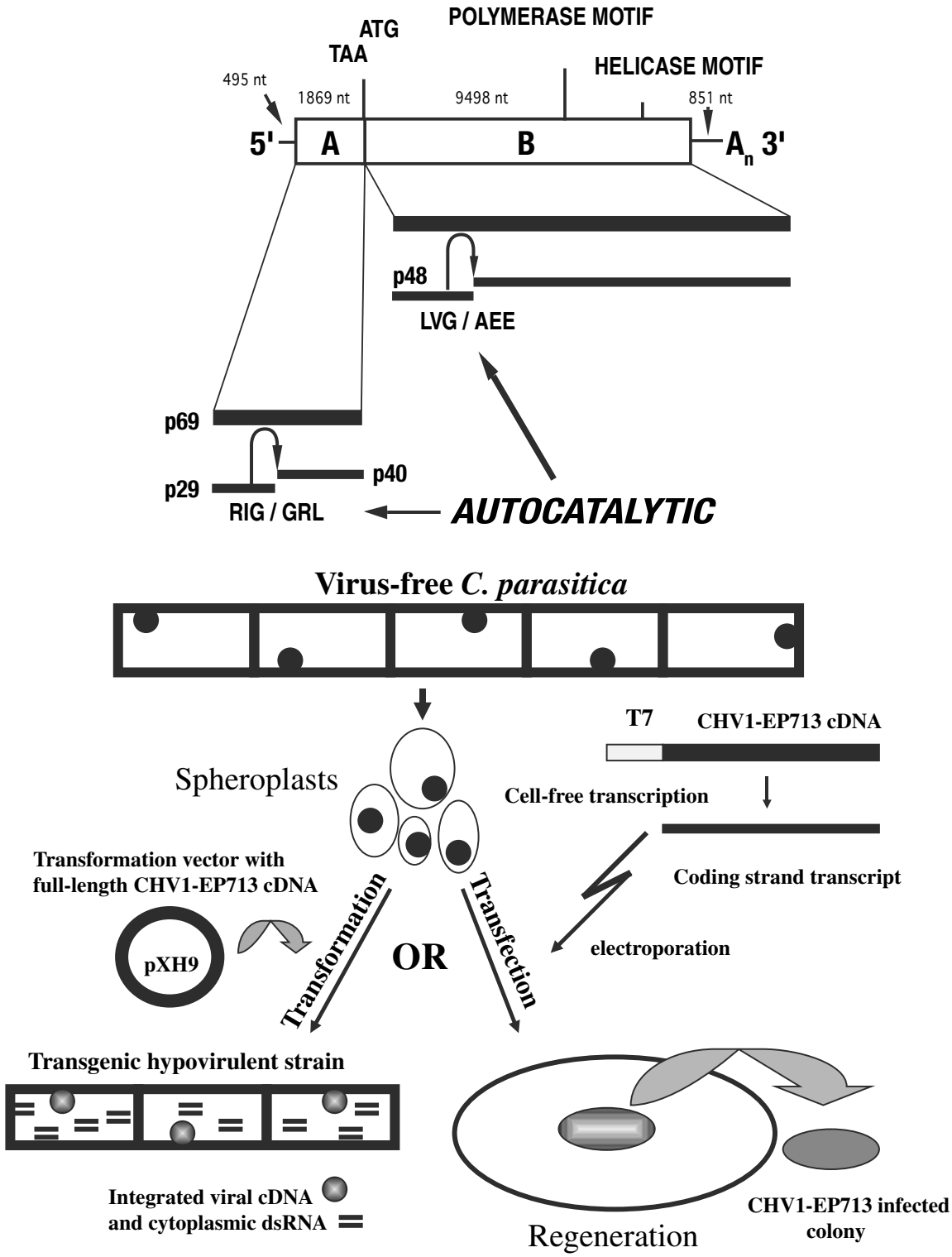

FIGURE 1 Hypovirus CHV1-EP713 genome organization, expression strategy, and reverse genetics. (A) The coding strand RNA of prototypic hypovirus CHV1-EP713 consists of 12,712 nucleotides (nt), excluding the poly(A) tail. The 5′-proximal coding domain, open reading frame A (622 codons), encodes two polypeptides, p29 and p40, that are released from a polyprotein, p69, by an autocatalytic event mediated by p29. Expression of open reading frame B (3,165 codons) also involves an autoproteolytic event in which a 48-kDa polypeptide, p48, is released from the N-terminal portion of the encoded polyprotein. Adapted with permission from Shapira et al., 1991. (B) Diagram illustrating the basic elements of the *C. parasitica* transformation (left) and transfection (right) protocols. Parallel horizontal bars (=) indicate hypovirus dsRNA. Adapted with permission from Nuss et al., 2002.

persistent, noncytopathic nature of hypovirus infections and the constellation of phenotypic changes that result from hypovirus infection, e.g., reduced pigment production, reduced asexual sporulation, reduced virulence, and loss of female fertility (reviewed by Nuss, 2005). The first experimental evidence supporting this view appeared in a report describing the reduced accumulation of the Gα subunit CPG-1 in hypovirus-infected *C. parasitica* and the substantial reduction in virulence that resulted from transgenic cosuppression of *cpg-1* expression (Choi et al., 1995). The importance of G-protein signaling in *C. parasitica* virulence was confirmed by targeted disruption of *cpg-1* (Gao and Nuss, 1996). Hypovirus-mediated reductions in the accumulation of both CPG-1 and the cognate Gβ subunit were subsequently shown to be regulated at the posttranscriptional level (Dawe et al., 2003). Transcriptional profiling studies on hypovirus-infected and G-protein subunit null mutant *C. parasitica* strains, using an expressed sequence tag-based microarray representing approximately 2,200 *C. parasitica* genes, identified a subset of G-protein-regulated, hypovirus-responsive genes (Dawe et al., 2003). These included a homologue of the yeast transcription factor Ste12. Subsequent analysis of the *cpst12* null mutant resulted in the identification of a subset (47) of Cpst12-regulated, hypovirus-responsive genes, providing a link between hypovirus-mediated modifications in cellular signaling and changes in the host transcriptional profile (Deng et al., 2007). A role for G-protein signaling in pathogenesis has now been established for most animal and plant pathogenic fungi (Li et al., 2007). Hypovirus-mediated alterations of calcium/calmodulin/inositol triphosphate-dependent (Larson et al., 1992) and mitogen-activated protein kinase signaling cascades (Park et al., 2004) have also been reported. The availability of the *C. parasitica* genome sequence will greatly facilitate future studies of the influence of hypovirus infection on host regulatory pathways and gene expression and allow a more precise understanding of their contribution to virus-mediated symptom expression.

FUNGAL ANTIVIRAL DEFENSE MECHANISMS

Animals and plants have elaborate mechanisms for defense against virus infection involving combinations of innate and adaptive responses that include RNA silencing, interferon production, and antibody production. Antiviral defense mechanisms currently identified in fungi include a self/nonself recognition system that presents barriers to the major mode of mycovirus transmission and an RNA recognition system that targets mycovirus RNA for destruction.

Vegetative Incompatibility

Vegetative incompatibility is a self/nonself recognition system that controls the ability of many fungi to undergo vegetative fusion events (anastomoses) that lead to cytoplasmic mixing and heterokaryon formation (reviewed by Glass and Kaneko, 2003; see also chapter 20). In the absence of an extracellular phase to their life cycle, mycoviruses rely on anastomosis as a major avenue for horizontal transmission. This has led to the long-held proposal that the genetic control of vegetative incompatibility may have evolved as a barrier to cytoplasmic infections by viruses, senescence plasmids, or transposable elements (Caten, 1972).

The vegetative incompatibility (*vic*) system in *C. parasitica*, which is controlled by at least six genetic loci with two alleles at each locus (Cortesi and Milgroom, 1998), clearly reduces transmission of mycoviruses between individuals in the laboratory (reviewed by Milgroom and Cortesi, 2004). High levels of *vic* diversity are thought to impose barriers to the spread of hypoviruses through wild *C. parasitica* populations, thereby reducing biocontrol efficacy. However, as noted by Milgroom and Cortesi (2004), low levels of *vic* diversity do not guarantee successful hypovirus spread through natural *C. parasitica* populations, indicating the influence of other contributing factors.

RNA Silencing

In fungi, mechanisms underlying RNA silencing, or RNA-mediated, sequence-specific suppression of gene expression, have been elucidated primarily through studies with *Neurospora crassa* (chapter 9). Since microRNAs, small RNAs that are produced in plants and animals from genome-encoded RNA hairpins and are involved in development and cellular regulation, have not been detected in fungi (Cerutti and Casas-Mollano, 2005; Nakayashiki et al., 2006), fungal RNA silencing has been portrayed as having originated as an ancient defense mechanism against invasive nucleic acids and viruses. Indeed, transposon silencing has been demonstrated in *N. crassa* (Nolan et al., 2005). However, the absence of a mycovirus experimental system has limited the use of *N. crassa* to examine whether RNA silencing also provides antiviral defense in fungi. Recent studies with hypovirus- and reovirus-infected *C. parasitica* and *Aspergillus* mycoviruses have demonstrated that RNA silencing also serves as an antiviral defense mechanism in fungi.

Viruses of plants and a growing number of animal viruses have been found to encode proteins that suppress host RNA silencing pathways (reviewed by Voinnet, 2005). Similarities between hypovirus-encoded protein p29 (Fig. 1) and the plant potyvirus-encoded suppressor of RNA silencing, HC-Pro (Choi et al., 1991), and reports that p29 could act in *trans* to increase the accumulation of homologous hypovirus RNA as well as heterologous mycoreovirus RNA (Sun et al., 2006) stimulated predictions that p29 might be a mycovirus-silencing suppressor. Segers et al (2006) tested this prediction and showed that p29 suppressed RNA silencing both in *C. parasitica* and in a heterologous plant system. Direct evidence that RNA silencing serves an antimycovirus role was subsequently provided by the demonstration that disruption of one of two *C. parasitica* dicer genes, *dcl-2*, the homologue of *N. crassa* dicer gene *dcl-2* (chapter 9), increased susceptibility to infections by hypovirus CHV1-EP713 and reovirus MyRV1-9B21 (Segers et al., 2007).

Hammond et al. (2008) observed that infection by *Aspergillus* virus 1816 resulted in suppression of inverted repeat transgene (hairpin) silencing. These authors also detected small RNA derived from *Aspergillus* virus 341 by Northern analysis, but only in an *A. nidulans* argonaute mutant strain, indicating that virus-derived small RNAs (vsRNAs) are present at a low level in some mycovirus-infected wild-type *Aspergillus* strains.

Details of vsRNA biogenesis in fungi are beginning to emerge. Zhang et al. (2008) demonstrated that hypovirus-derived vsRNAs accumulated in wild-type and *dcl-1* mutant *C. parasitica* strains, but not in the *dcl-2* mutant strain that was previously shown to have increased susceptibility to hypovirus infection. Cloning and sequence analysis of the hypovirus vsRNAs showed that they were produced from both positive and negative viral RNA strands in a 3:2 ratio and in a nonrandom distribution along the viral genome. A significant portion (>50%) of the hypovirus-specific vsRNAs were found to contain a 3′ terminal mismatch with the viral RNA sequence. In most cases, the mismatch

consisted of a single or double adenosine. Similar terminal mismatches, which may result from the addition of non-template-encoded residues, have not been reported for vsRNAs generated from plant or animal virus RNAs. It will be interesting to see whether small-interfering RNAs generated in uninfected fungi also contain 3′-terminal mismatches when that information becomes available.

Consistent with the requirement of C. *parasitica dcl-2* for biogenesis of hypovirus vsRNAs, Zhang et al. (2008) reported that *dcl-2* transcript accumulation increased 10- to 15-fold in response to hypovirus or mycoreovirus infections, while the expression of *dcl-1* increased only modestly (2-fold). Interestingly, *dcl-2* transcript levels were found to be superinduced (35-fold) following infection by a mutant hypovirus that lacked the p29 suppressor of RNA silencing. In this regard, Choudhary et al. (2007) recently showed that the production of hairpin dsRNA in *N. crassa* results in induction of both argonaute *qde-2* and dicer *dcl-2* expression. Interestingly, Hammond et al. (2008) observed no change in *A. nidulans* argonaute (*rsdA*) or dicer (*dclB*) transcript levels after infection by any of three *Aspergillus* mycoviruses.

The prospects for using mycovirus experimental systems for elucidating mechanisms underlying the induction and suppression of antiviral RNA silencing are promising due to simplified RNA silencing pathways and the evolutionary position of fungi relative to plants and animals. Additionally, the C. *parasitica*/mycovirus experimental system takes advantage of a highly tractable eukaryotic host that is the natural host to a constellation of well-characterized viruses with different replication strategies representing five different virus taxonomic families and a facile hypovirus reverse genetics capability. In this regard, a novel role for the C. *parasitica* dicer DCL-2 in viral RNA recombination was recently reported (Zhang and Nuss, 2008).

PROSPECTS FOR ENGINEERING MYCOVIRUSES TO MANIPULATE THE FUNGAL HOST

The concept of engineering mycoviruses to manipulate the phenotypic traits of the fungal host has, in fact, been reduced to practice. The installation of a full-length infectious hypovirus cDNA copy into C. *parasitica* chromosomal DNA has provided a novel means of hypovirus transmission not observed in nature (reviewed by Nuss, 2005). In contrast to hypovirus RNA, which is not transmitted to ascospores, the hypovirus cDNA copy that is present in the nucleus of transgenic hypovirulent strains is inherited by a portion of the ascospore progeny, followed by generation of cytoplasmically replicating viral RNA. Because of allelic rearrangement at the vegetative incompatibility loci during meiosis, the hypovirulent ascospore progeny represent a spectrum of different *vic* types. The prediction that this novel mode of transmission will facilitate cytoplasmic spread of hypoviruses in natural C. *parasitica* populations is currently being tested.

The hypovirus transfection and transformation protocols have provided the means to extend hypovirus host range, allowing the engineering of hypovirulent strains of four fungal species that are closely related to C. *parasitica* (Chen et al., 1994b) and two fungal tree pathogens in separate genera (Sasaki et al., 2002). It has also been possible to engineer chimeras of mild and severe hypovirus isolates to fine-tune the interaction between C. *parasitica* and its plant host (Chen et al., 2000).

The potential for using mycoviruses to manipulate fungal phenotype extends past hypovirulence. The ability of Curvularia thermal tolerance virus-infected *Curvularia protuberata* to confer heat tolerance in tomato as well as the natural panic grass host provides a prime example (Marquez et al., 2007). The reduced production of trichothecene mycotoxins by the *F. graminearum* virus FgV-DK21 provides another (Kwon et al., 2007). Clearly, many exciting opportunities for engineering mycoviruses as tools for studying and manipulating fungal hosts are anticipated with advances in the development of corresponding mycovirus reverse genetics and fungal transformation protocols.

SUMMARY

Mycoviruses are now known to be widely distributed throughout the kingdom Fungi. Although most mycovirus infections appear to be asymptomatic under laboratory conditions, the effect of these persistent virus infections on the fungal host over time in nature is not well understood and deserves more intense scrutiny. Detailed molecular characterizations have, justifiably, been conducted primarily for those mycoviruses that cause interesting phenotypic changes in the fungal host, e.g., hypovirulence. These analyses are revealing an increasing number of unclassified viruses, exposing the need for significant adjustments to the current mycovirus taxonomic structure. Advances made with the hypovirus-C. *parasitica* experimental system have demonstrated that, similar to viruses of plants and animals, mycoviruses have utility for elucidating host function and manipulating host phenotype. It is anticipated that the development of similar capabilities for other mycoviruses will provide access to a rich repertoire of biological interactions for fundamental and practical application.

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ORGANELLES

III

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Mitochondria and Respiration

FRANK E. NARGANG AND JOHN C. KENNELL

Mitochondria are double-membrane-bound organelles that are divided into four subcompartments: the mitochondrial outer membrane (MOM), the intermembrane space (IMS), the mitochondrial inner membrane (MIM), and the matrix. Mitochondria grow by the incorporation of newly synthesized material into existing organelles that eventually divide. They contain their own DNA (mtDNA), which specifies a few proteins involved in oxidative phosphorylation. However, the vast majority of proteins in mitochondria are encoded by nuclear genes, synthesized on cytoplasmic ribosomes, and imported into the organelles. Many crucial cellular activities, such as the biogenesis of iron-sulfur clusters, apoptosis, and calcium homeostasis, involve mitochondria. However, the best-known function of mitochondria is ATP production via cellular respiration. This chapter covers the functions of mitochondria in filamentous fungi.

CYTOLOGY AND INHERITANCE

Confocal microscopy images of mycelia stained with mitochondrion-specific dyes reveal mitochondria as elongated tubes that are densely packed in a parallel orientation at the growing tip of hyphal cells. Mitochondria in subapical regions are more sparsely distributed and tend to be shorter and more rod-shaped. While branched mitochondria are common in yeast (Okamoto and Shaw, 2005), they are not readily detected in filamentous fungi (Hickey et al., 2004). Mitochondria are highly dynamic and readily move from cell to cell through septal pores. Movement is directed by interactions with actin filaments and/or microtubules, depending on the organism (Boldogh and Pon, 2006; Fuchs et al., 2002). Images of cytoplasmic flow following anastomosis show that mitochondria are rapidly transferred through the fusion pore with the direction of flow thought to be related to differences in the turgor pressure between hyphal cells involved in fusion (Hickey et al., 2002). The use of potentiometric dyes, such as rhodamine 123, provides a measure of the MIM potential, and staining intensities of growing hyphal cells show that mitochondria at the growth tip are highly active. While this appears to reflect the high energy demands associated with apical growth, one study suggests that mitochondria at the growing tip consume little oxygen and may play a role in calcium sequestration (Levina and Lew, 2006).

In fungi that have morphologically distinct gametes (i.e., anisogametic), mitochondria are inherited via the maternal parent. In heterothallic filamentous ascomycetes that develop fruiting bodies, the parent that forms the sexual fruiting body contributes mitochondria to the ascogenous cell. Although mitochondria of the conidial parent are generally excluded, several incidences of paternal mtDNA transmission have been reported (reviewed by Yan and Xu, 2005). The most direct mechanism of transmission would seem to occur via leakage of the male mitochondria into the sexual hypha, the trichogyne. An alternative route may also occur during the development of the fruiting body in perithecium-forming fungi (Griffiths, 1995). Crosses between strains that are vegetatively compatible versus those that are incompatible showed that the rates of paternal inheritance of mitochondrial plasmids (mt plasmids) (described below) was 10-fold higher among compatible strains, suggesting that plasmids enter the maternal tissue via fusion of cells destined to form the ascogenous cell, rather than via fusion of conidia and the trichogyne (Debets and Griffiths, 1998). Furthermore, studies with *Aspergillus nidulans* showed that cytoplasms of compatible parents mix prior to the formation of the cleistothecia, whereas those of incompatible parents do not (Bruggeman et al., 2003).

THE ELECTRON TRANSPORT CHAIN

Mitochondrial respiration occurs via transfer of electrons from reduced electron carriers to molecular oxygen. This is accomplished using an electron transport chain housed in the MIM (Fig. 1). Four large enzyme complexes are involved in the transfers, and three of these (complexes I, III, and IV) have the ability to couple electron transfer to the pumping of protons across the MIM from the matrix to the IMS. Complex V, the ATP synthase, utilizes the energy stored in the proton gradient to synthesize ATP. Most current information on these complexes has come from studies

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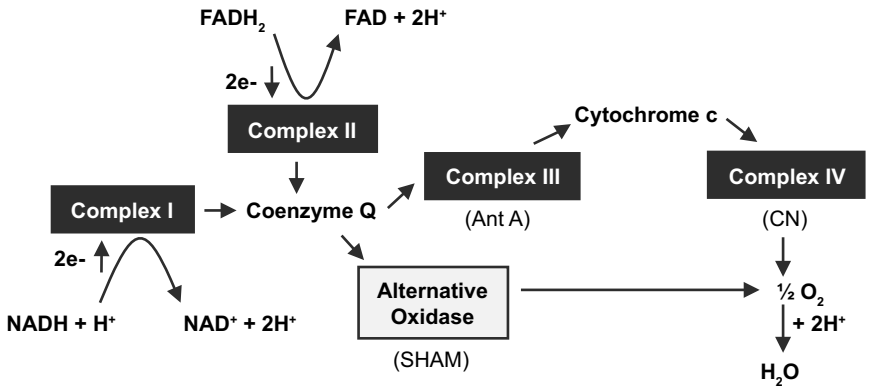

FIGURE 1 Electron transport chain. Electrons from the reduced electron carriers NADH + H^+ and $FADH_2$ are extracted by complex I and complex II, respectively, and transferred through the electron transport chain in the direction of the arrows. Complex IV uses the electrons to reduce molecular oxygen to water. The four large enzyme complexes are shown as black boxes. The pathway that ends at complex IV is referred to as the cytochrome-mediated electron transport chain (cmETC) in this chapter. At coenzyme Q (the ubiquinone/ubiquinol pool) the path to oxygen may branch in organisms capable of producing AOX. Complexes I, III, and IV are sites where electron transfer is coupled to proton pumping. The cmETC refers to the path of electrons from complex I to oxygen via complex IV since cytochromes *b* and c_1 (found in complex III), cytochrome *c*, and cytochromes *a* and a_3 (found in complex IV) are involved in electron transfer via this path. Chemicals mentioned in the text that inhibit electron flow are shown in parentheses under the enzymes that they affect (Ant A, antimycin A; CN, cyanide; SHAM, salicylhydroxamic acid).

on mammalian tissues or from *Saccharomyces cerevisiae*. However, *S. cerevisiae* does not contain complex I, and *Neurospora crassa* has provided a useful model for the study of the structure and function of this complex.

Respiratory Complex I

Complex I has NADH:ubiquinone oxidoreductase activity and couples the transfer of electrons to the pumping of protons from the matrix to the IMS. The *N. crassa* enzyme contains at least 39 protein subunits as well as flavin mononucleotide and eight to nine iron sulfur clusters. Seven of the subunits are encoded on mtDNA. The enzyme exists as an L-shaped structure with the membrane arm integrated into the MIM and a peripheral arm associated with the membrane arm that protrudes into the matrix (Marques et al., 2005; Videira and Duarte, 2002). Although the location of all subunits within the complex is not known with certainty, it has been predicted (Duarte and Videira, 2007) that 16 subunits exist in the membrane arm and 23 subunits are in the peripheral arm (Fig. 2). It has been shown that in *N. crassa* the enzyme is not essential for growth but is required for successful completion of the sexual phase (Duarte et al., 1998).

Work with *N. crassa* showed that large and small intermediates of the membrane arm assemble independently and then join to yield the fully assembled subcomplex. Based on the assembly phenotypes of several complex I subunit mutants, it has recently been suggested that the large intermediate of the membrane arm itself is first formed from two other smaller subcomplexes (Duarte and Videira, 2007). The peripheral arm also assembles independently, and when both arms are complete, they associate to form functional complex I (Nehls et al., 1992; Tuschen et al., 1990). Examination of complex assembly in knockout mutants lacking specific subunits revealed that the loss of certain subunits has no effect on the assembly of the other subunits of the complex, while loss of other subunits leads to assembly defects (Schulte, 2001; Videira, 1998). As expected, the loss of a protein subunit required for assembly of one arm of the enzyme normally does not affect assembly of the other arm. However, there are exceptions to this rule. In *N. crassa*, an acyl carrier protein traditionally associated with fatty acid synthesis is also a subunit of the complex I peripheral arm. Loss of acyl carrier protein results in defects in assembly of both the peripheral and membrane arms, although the mechanism by which the membrane arm is affected is not well understood (Schneider et al., 1995; Schulte, 2001). Similarly, loss of an 11.5-kDa subunit of the membrane arm leads to defects in assembling both the membrane and peripheral arms. It was suggested that the 11.5-kDa protein may participate in connecting the two arms of the complex and that subunits of the peripheral arm might degrade or aggregate when it is absent (Marques et al., 2005, 2007).

Recently, it has been shown that *N. crassa* complex I mutants are more resistant than wild-type strains to phytosphingosine (Castro et al., 2008), a drug that was previously shown to induce apoptosis-like death in *A. nidulans* (Cheng et al., 2003). Interestingly, mutants affecting other respiratory complexes were more sensitive to the drug, and it was suggested that complex I plays a direct role in apoptosis. Furthermore, it has been shown that caspase cleavage of the 75-kDa subunit of complex I in mammals is necessary for various changes that occur in mitochondria during apoptosis (Ricci et al., 2004). Additional relationships between fungal and mammalian apoptosis are evident from studies of other mitochondrial proteins. A mutant of the *N. crassa* homologue of the mammalian apoptosis-inducing factor was also more resistant to phytosphingosine than the wild type. A deletion of the gene encoding the *N. crassa* AMID-like protein, another protein known to be involved in mammalian apoptosis, resulted in increased sensitivity to

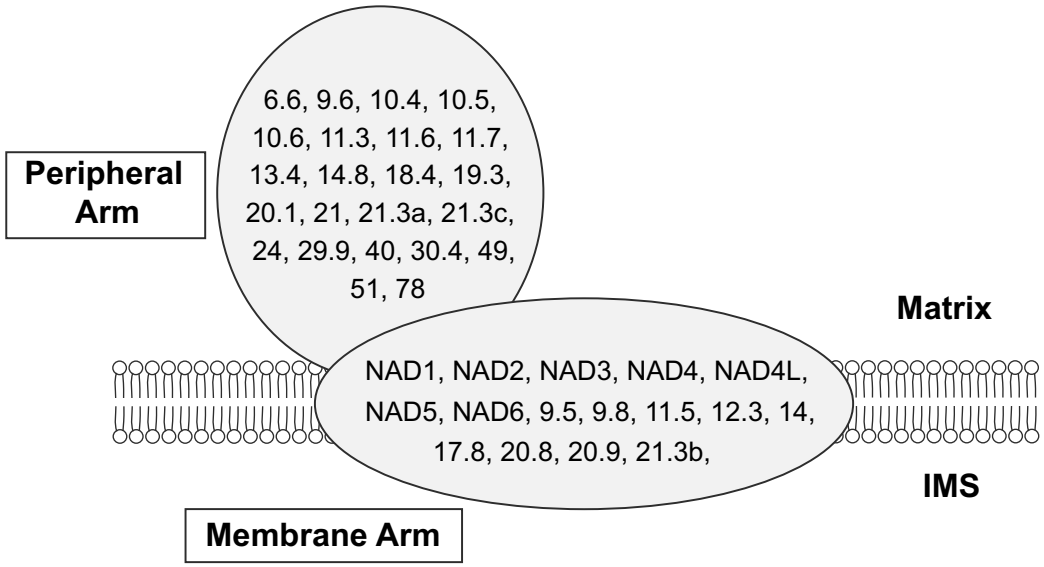

FIGURE 2 Predicted placement of subunits in the peripheral or membrane arms of *N. crassa* complex I (Duarte and Videira, 2007). The seven mitochondrially encoded subunits are referred to as NAD proteins but may be called ND proteins in some other references. The subunits encoded within the nucleus are referred to according to their molecular mass in kilodaltons.

phytosphingosine (Castro et al., 2008). These findings suggest that filamentous fungi can serve as useful models for apoptosis in higher organisms.

Alternative NADH or NADPH Dehydrogenases

In addition to complex I, fungi and plants also contain single polypeptide dehydrogenases, which oxidase either NADH, NADPH, or both [symbolized as NAD(P)H]. They donate electrons to the electron transport chain but are incapable of proton pumping. In *N. crassa* there are four such enzymes. NDE1 [NAD(P)H dehydrogenase external 1] is an NADPH dehydrogenase (Melo et al., 2001), while NDE2 uses both NADH and NADPH as substrates (Carneiro et al., 2004). Both enzymes are anchored to the MIM by a single membrane-spanning domain with their catalytic activity present in the IMS. Thus, these enzymes oxidize substrates external to the matrix. NDE3 also oxidizes both NADH and NADPH and is found as a soluble protein in both the mitochondrial IMS and the cytosol (Carneiro et al., 2007). The fourth enzyme of this class in *N. crassa* is NDI1 [NAD(P)H dehydrogenase internal 1]. This protein was found to be a peripheral membrane protein on the inner side of the MIM that oxidizes NADH within the matrix (Duarte et al., 2003). The existence of these enzymes likely explains why *N. crassa* mutants with nonfunctional complex I are still viable. Triple knockouts of *nde1*, *nde2*, and *nde3* are also viable, suggesting that there are other systems in *N. crassa* that oxidize cytoplasmic pools of NAD(P)H (Carneiro et al., 2007).

Double mutants of *ndi-1* and any subunit of complex I that eliminates its activity are lethal, since both systems of oxidizing matrix NADH are nonfunctional. Although single *ndi-1* mutants have no effect on vegetative growth, germination from both conidia and ascospores is much slower than that from wild-type spores (Duarte et al., 2003). Double mutants of either *nde-1* or *nde-3* and complex I are viable. Since NDE2 is active in the IMS, it was surprising that *nde-2*/complex I double mutants were inviable. It was suggested that *N. crassa* may be capable of exchanging matrix and external NADH so that NDE2 may complement the activity of complex I (Carneiro et al., 2004). Taken together these results indicate that the single-enzyme NAD(P)H dehydrogenases play an important role in the growth and development of *N. crassa*.

THE MITOCHONDRIAL GENOME

Fungal mitochondrial genomes are AT-rich DNAs that map as circular molecules. However, several studies suggest that fungal mtDNAs are actually long, concatenated linear molecules (reviewed by Nosek and Tomaska, 2003). True monomer-length linear mitochondrial genomes have also been found in a number of ascomycetes, and a variety of telomeric structures have been identified. These include covalently closed terminal hairpins and tandemly repeated sequences of 739 bp or larger. Although common in plants, segmented mtDNAs are rare in fungi. Thus far, the only segmented genome identified is in the chytridiomycete *Spizellomyces punctatus*, which is composed of three circular molecules (Laforest et al., 1997).

The sizes of fungal mtDNAs reported to date range from 18 to >150 kb (reviewed by Bullerwell and Lang, 2005; Hausner, 2003; and Kennell and Cohen, 2003). The size extremes fall between the average size of animal mtDNAs and the lower limit of plant mtDNAs. Despite the size differences, most fungal mitochondrial genomes encode the same set of 13 polypeptides found in animal mitochondrial genomes (Burger et al., 2003). The set includes apocytochrome *b*; cytochrome oxidase subunits 1, 2, and 3; complex I (NADH dehydrogenase) subunits 1, 2, 3, 4, 4L, 5, and 6; and ATPase subunits 6 and 8. In addition, many fungal mitochondrial genomes also encode ATPase subunit 9 and a ribosomal protein (*rps3*; [Bullerwell et al., 2000; Bullerwell and Lang, 2005]). Notably, *Saccharomyces* and *Schizosaccharomyces* species have lost the genes for NADH dehydrogenase. The genes carried on *N. crassa* mtDNA are

TABLE 1 *N. crassa* mitochondrial genes[a]

Gene	Product	Subunit of:
nad1	NAD1	Complex I
nad2	NAD2	Complex I
nad3	NAD3	Complex I
nad4	NAD4	Complex I
nad4L	NAD4L	Complex I
nad5	NAD5	Complex I
nad6	NAD6	Complex I
cob	COB	Complex III
cox1	COX1	Complex IV
cox2	COX2	Complex IV
cox3	COX3	Complex IV
atp6	ATP6	Complex V
atp8	ATP8	Complex V
atp9	ATP9	Complex V
rnl	25S rRNA	Ribosome
rns	19S rRNA	Ribosome
rps3 or *S5*	Ribosomal protein S3	Ribosome
28 tRNAs	28 tRNAs	

[a]Data from Kennell et al., 2004, and Cahan and Kennell, 2005. In some references the *nad* genes and NAD proteins are referred to as *nd* and ND, respectively.

shown in Table 1 as representative of a "typical" filamentous fungus.

Although the genetic code used in the expression of mitochondrial encoded genes can differ both within and between fungal phyla, filamentous ascomycete species annotated to date use the codon UGA to specify tryptophan, rather than a stop codon. In addition to protein-encoding regions, mitochondrial genomes also encode large and small rRNAs as well as a minimal set of tRNAs needed for translation. Interestingly, chytridiomycete fungi have lost a large number of tRNAs and presumably import the necessary tRNAs from the cytoplasm. In other fungal phyla, mtDNAs occasionally lack a few tRNAs necessary to accommodate all codons represented among mitochondrion-encoded genes. In this case, either the tRNAs are imported or tRNAs having U residues at the third anticodon position may undergo a superwobble to read all four nucleotides in the third codon position (Rogalski et al., 2008). In addition to rRNAs and tRNAs, several ascomycetes encode a gene for the mitochondrial RNase P RNA subunit (*mpB*) (Bullerwell and Lang, 2005).

The standard set of fungal mitochondrial genes resembles that of animals and is believed to represent independent gene loss in the two lineages from a common protist ancestor (Bullerwell and Lang, 2005). What differentiates fungal from animal mtDNAs is the amount of noncoding regions and tolerance of selfish genetic elements such as mobile introns and plasmids (see below). The intron content of fungal mtDNAs varies widely, constituting from zero to over 50% of individual genomes. Mitochondrial introns fall into two discrete classes, group I and group II, which differ in both structure and splicing mechanism (see below). Fungal mtDNAs also contain a variety of repetitive sequences. These repetitive elements are variable in length but are usually less than 100 bp. The majority are palindromes that have conserved secondary structures, such as "G+C clusters" in *Saccharomyces* spp. (de Zamaroczy and Bernardi, 1986), "double-hairpin elements" in *Allomyces macrogynus* (Paquin et al., 2000), and "PstI palindromes" and "CG-rich palindromes" in *N. crassa* (Cahan and Kennell, 2005; Yin et al., 1981). The number of repeats within the mtDNA of a particular species varies depending on the strain examined (Cahan and Kennell, 2005). Smaller, nonpalindromic repeats have also been detected; for example, there are 124 copies of an 11-bp repeat in the 100-kb genome of *Podospora anserina* (Koll et al., 1996). The distribution pattern of many of these elements suggests that they are mobile (Cahan and Kennell, 2005; Paquin et al., 2000). Fungal mitochondria also can harbor extrachromosomal DNA plasmids (described below), and plasmid-derived sequences are commonly detected in mtDNAs.

MITOCHONDRIAL SELFISH GENETIC ELEMENTS

Mobile Introns

The study of fungal mitochondrial introns has been an active area of research that has yielded a number of significant findings. Notably, the study of mechanisms of splicing and intron mobility of group I and group II introns was initially carried out with *S. cerevisiae* and was later expanded to other fungi, plant mitochondria and chloroplasts, and more recently to bacteria. Among the findings described below and elsewhere is speculation that these elements evolved in the RNA world (Gilbert and de Souza, 1999) and played an important role in the evolution of organelle and nuclear genomes alike. For instance, it is increasingly accepted that group II introns were ancestors of spliceosomal introns (reviewed by Lambowitz and Zimmerly, 2004, and Rodriguez-Trelles et al., 2006). Recently, it was suggested that the introduction of mobile group II introns into the primordial eukaryotic cell via the alphaproteobacterial ancestor of mitochondria could have provided the driving force that led to the evolution of the nuclear membrane (Lopez-Garcia and Moreira, 2006; Martin and Koonin, 2006).

Group I Introns

Among the first group I introns identified were those found in fungal mtDNA. A recently developed algorithm is able to separate group I introns into six categories based on conserved intron core sequences (Lang et al., 2007). In vitro studies show that group I intron RNAs are ribozymes, capable of self-catalysis; however, in vivo, group I introns are commonly associated with proteins that facilitate splicing. In some cases, the mitochondrial proteins involved in splicing are bifunctional and are involved in other essential functions. In *N. crassa*, a mitochondrial tyrosyl-tRNA synthetase has adapted to function in group I intron splicing (Akins and Lambowitz, 1987). The synthetase protein, encoded by the nuclear *cyt-18* locus, both aminoacylates mitochondrial $tRNA^{Tyr}$ and promotes group I intron splicing by stabilizing the catalytically active RNA structure (Caprara et al., 1996; Mohr et al., 1992). Molecular and biochemical studies, coupled with the crystal structure of the CYT18-group I intron RNA, demonstrate that the protein binds to the intron by using a surface that is separate and distinct from that which binds $tRNA^{Tyr}$ (Paukstelis et al., 2008). Comparative genomic analysis suggests that the adaptation of CYT-18 protein that created a separate domain associated with splicing evolved during or after the divergence of the subphylum Pezizomycotina from Saccharomycotina

(Paukstelis and Lambowitz, 2008). The evolution of a new binding site associated with group I splicing also appears to have occurred with an intron-associated gene, I-AniI, of *Aspergillus*, whose intron open reading frame (ORF) product also participates in intron mobility (Caprara and Waring, 2005). Another host factor involved in group I intron splicing was identified in *N. crassa*—the product of the *cyt-19* gene (Mohr et al., 2002). In this case, the CYT19 protein functions as an ATP-dependent RNA chaperone to facilitate both group I and group II intron splicing. CYT19 is an RNA helicase (i.e., DExH/D-box protein) that appears to destabilize inactive structures that act as kinetic traps in RNA folding (Halls et al., 2007).

In addition to secondary and tertiary structural characteristics, group I introns can also be distinguished by ORFs contained within the intron. Most commonly, ORFs associated with group I introns encode endonucleases that are involved in intron mobility. The endonucleases typically recognize a 15- to 45-bp DNA sequence of an intronless allele of the gene in which they reside. The transposition event is therefore site specific and described as "homing." Insertion of group I introns/homing endonuclease genes is initiated by a double-stranded cut in the recipient allele catalyzed by the endonuclease. This stimulates the double-strand break repair response of the host, and the donor allele is used as a template for DNA synthesis, resulting in a copy of the intron being inserted into the recipient allele.

There are four families of homing endonucleases (HEs), two of which have been found in fungal mtDNAs, i.e., "LAGLIDADG" and "GIY-YIG," with the letters indicating conserved amino acid sequences (Chevalier and Stoddard, 2001). The LAGLIDADG HEs can also be further divided according to whether they have one or two motifs (Haugen et al., 2005). The HE ORFs either are freestanding in the intron or can be in frame with the upstream exon. In the latter case, a proteolytic cleavage may be involved to liberate the active protein (van Dyck et al., 1998). Homing endonuclease genes are believed to be independent selfish genetic elements in their own right (reviewed by Haugen et al., 2005), and their association with group I (and group II) self-splicing introns was a beneficial union for both parties, as the intron minimized damage to the host by splicing the element out of RNAs prior to translation while the HE facilitated mobility.

Several intron-encoded HEs are bifunctional and participate in splicing (called maturases), like the aforementioned I-AniI gene. HEs that have maturase activity are more likely to be retained, as they enable host genes to be properly spliced. However, there are many examples of intron-encoded genes that are truncated or absent altogether. These introns are presumed to have lost the ability to transpose (Haugen et al., 2005) and are usually dependent on proteins encoded by another intron (Henke et al., 1995) or by host factors (Lambowitz and Perlman, 1990) for splicing. It is interesting that most filamentous ascomycetes contain a group I intron in the large rRNA gene (*rnl*). Rather than encoding an HE as is the case in yeasts, the intron carries a gene for protein subunit 3 of the small ribosome (*rps3*). By carrying an essential host gene, the intron is maintained and thus is highly conserved.

Group II Introns

Group II introns were initially identified in fungal mtDNAs and the organellar genomes of plants (Michel et al., 1989). While they have a broad distribution, group II introns occur infrequently in fungal mitochondrial genomes. They can be divided into three families, based on predicted secondary structure (types IIA, IIB, and IIC) with fungal mitochondrial group II introns belonging to IIA (Toro et al., 2007). Like group I introns, they are ribozymes, but splicing is protein assisted in vivo. The group II splicing mechanism is similar to that of spliceosomal introns, supporting the hypothesis that group II introns are the predecessors to nuclear spliceosomal introns (Pyle and Lambowitz, 2006).

Mitochondrial group II introns also contain ORFs that encode proteins involved in mobility and/or splicing. These group II intron-encoded proteins are multifunctional and display a remarkable number of biochemical activities. For instance, those found in fungal mitochondria function as a reverse transcriptase (RT), an RNA maturase, a DNA binding protein, and an endonuclease (reviewed by Lambowitz and Zimmerly, 2004). Since their initial identification in fungal mitochondria and chloroplasts of higher plants, group II introns have been detected in both eubacteria and archeabacteria. Although rare in the archaea, group II introns are surprisingly common in both gram-positive and gram-negative bacteria, occurring in approximately 25% of genomes that have been sequenced (Toro, 2003).

Unlike the DNA-mediated transposition of group I introns, group II mobility occurs via an RNA intermediate and involves reverse splicing of the liberated intron RNA into the DNA of the intronless allele, followed by the reverse transcription of inserted intron RNA. This process is site specific and typically involves an H-N-H (his-asn-his) HE domain of the intron-encoded protein, which defines a third family of HEs. The specificity for the insertion site is primarily dictated by sequences in the intron RNA. This makes it possible to target mobile group II introns to insert into different sites in DNA simply by modifying intron RNA sequences. Programmable gene-targeting vectors, called "targetrons," have been developed and can be used in bacteria for targeted gene disruption (reviewed by Lambowitz et al., 2005). These vectors can also be used to knock in genes by the introduction of a cargo gene into a specific domain of the intron. Efforts are under way to use targetrons in eukaryotic genomes. If successful, they could become an important tool for use in genetic engineering and gene therapy (Pyle and Lambowitz, 2006; Toro et al., 2007).

mt Plasmids

Fungal mitochondria frequently contain extrachromosomal DNAs (and in some cases RNAs), and these genetic elements can be divided into two distinct groups: (i) plasmid-like mitochondrial elements (plMEs) that derive from the mitochondrial genome and (ii) naturally occurring, autonomously replicating plasmids that have little or no homology to mtDNA. Members of the first group are circular, oligomeric DNAs that are homologous to regions of the mitochondrial genome (reviewed by Hausner, 2003). The second group contains "true" plasmids, which exist in several forms that can be classified based on their structure (linear or circular) and by the types of nucleic acids directly involved in their replication: DNA, RNA, or retroplasmids (i.e., those that have both RNA and DNA replicative forms; reviewed by Griffiths et al., 1995).

plMEs Derived from mtDNA

The most thoroughly studied group of plMEs are the so-called p^- mtDNAs that define one class of the *petite* respiratory-defective mutants of *S. cerevisiae*. The p^- mtDNAs are deleted forms that retain anywhere from 35 bp to approximately one-third of the wild-type mitochondrial genome (Dujon, 1981). The portion that remains is amplified back up

to the size of the wild-type mtDNA as a series of tandem repeats. Since p^- strains lack significant portions of mtDNA, they are dependent on fermentative pathways of energy production for survival. Some of these p^- mtDNAs are hypersuppressive and rapidly replace wild-type mtDNA in a population. They are thought to have a replicative advantage over wild-type mtDNA molecules (MacAlpine et al., 2001).

plMEs are detected in several obligate aerobes, such as *Aspergillus*, *Neurospora*, and *Podospora* species. Interestingly, they are usually detected only in strains that have mitochondrial defects associated with either nuclear or mitochondrial mutants. Consequently, strains containing plMEs are associated with growth defects, such as a "ragged" phenotype in *Aspergillus amstelodami*, a "stop-start" phenotype in *N. crassa*, and senescence in *P. anserina*. Multimeric plMEs have also been detected in the plant pathogens *Ophiostoma novo-ulmi* and *Cryphonectria parasitica*, where they are associated with degenerative disease and hypovirulence, respectively (Abu-Amero et al., 1995; Monteiro-Vitorello et al., 1995). The mechanisms associated with the formation of mtDNA-derived elements appear to vary between host genera.

In *P. anserina,* plMEs form during senescence, which for this fungus is a genetically programmed phenomenon (see below). As cultures age, multimeric circular DNAs accumulate and are associated with a concomitant deletion of regions of the mtDNA. Collectively called senDNAs, the most common type (α-senDNA or plDNA) has a monomer length of 2.5 kb and corresponds precisely to the first intron of the mitochondrial *cox1* gene. It is thought to be produced either by reverse transcription of the *cox1* pre-mRNA or by excision of the intron from the mtDNA (Jamet-Vierny et al., 1997; Sainsard-Chanet et al., 1993). Additional DNAs (e.g., β- or γ-senDNAs) are often detected in presenescent cultures, and these vary in both size and extent. Members of each class of senDNA share a common core sequence and appear to derive from recombination between short direct repeats of the mitochondrial genome (Cummings et al., 1985; Osiewacz and Scheckhuber, 2002). It was originally believed that senDNAs were responsible for mitochondrial impairment and growth decline, yet subsequent studies showed that some strains undergo senescence without the accumulation of senDNAs and that senDNAs were found to persist in some long-lived mutant strains (Silar et al., 1997; Silliker and Cummings, 1990), indicating that other mechanisms are likely involved (Jamet-Vierny et al., 1999). The prevailing view is that these senDNAs play a role in accelerating senescence but are not the causal agents (Osiewacz and Scheckhuber, 2002).

Several different plasmid-like mitochondrial elements have been found in *N. crassa*. Like the *petite* and senDNAs, they are multimeric DNAs that derive from relatively short regions of the mtDNA. It has been speculated that they originate from recombination events (Almasan and Mishra, 1990; Gross et al., 1989) or as a consequence of DNA repair due to stalled replication associated with the high-copy-number PstI palindromes (Hausner et al., 2006a). Some plMEs share a core consensus sequences, yet collectively they lack conserved regions that might serve as replication origins and appear to propagate by a rolling-circle method of replication (Hausner et al., 2006b). Interestingly, several plMEs contain the promoter sequence of the large rRNA gene (Gross et al., 1989; Hausner et al., 2006b) and are abundantly transcribed, and it has been hypothesized that the transcripts serve as primers for second-strand synthesis. Although the *Neurospora* plMEs arise in respiratory-deficient or UV-sensitive strains, it has been shown that they can be transferred to wild-type strains via anastomosis and propagated (Hausner et al., 2006b).

Naturally Occurring Plasmids

Naturally occurring, or true mt plasmids show no significant homology to mtDNA and replicate independently. They are widely distributed in filamentous fungi, and in *Neurospora* species it has been estimated that >50% of natural isolates contain one or more types of mt plasmid (Arganoza et al., 1994; Griffiths, 1995). In organisms other than filamentous fungi, mt plasmids occur infrequently. Although they are found in *Pichia* species, they have not been detected in other yeasts (Blaisonneau et al., 1999). They also show limited occurrence among basidiomycetes and have not been reported in zygomycete or chytridiomycete species.

The linear mt plasmids that use DNA or RNA in their mode of replication have an evolutionary relationship with cytoplasmic mycoviruses and may have been introduced into mitochondria recently, while the origin of linear and circular retroplasmids is thought to precede that of mitochondria (i.e., they arose prior to the introduction of the alphaproteobacterium-like organism into the primitive eukaryotic cell). Thus, they have been described as "molecular fossils," which are defined as contemporary genetic elements that are ancient in origin and provide insight into the evolutionary past (Maizels and Weiner, 1993). At first glance, mt plasmids seem to be similar to their bacterial counterparts, yet they differ in several important ways: (i) mt plasmids are more like bacteriophages in that they encode their own polymerase(s); (ii) while plasmid sequences are commonly detected within the mitochondrial chromosome, mt plasmids do not encode integrase genes and integration is not part of the replication cycle; (iii) mt plasmids do not provide an obvious selective advantage to their host (except in rare instances); and (iv) despite their widespread occurrence and potential as vehicles of transmitting genetic information, mt plasmids have not successfully been employed as vectors due to the inability to readily transform mitochondria.

Linear double-stranded DNA (dsDNA) mt plasmids that use a direct DNA replication mode are the most common type of plasmid in fungal mitochondria. They range in size from 2 kb to almost 10 kb and are described as "invertrons" because they have terminal inverted repeats and contain a 5′ covalently-linked terminal protein (Sakaguchi, 1990). The prototypical fungal linear DNA plasmid contains two large ORFs, one on each strand, directed inward from the terminal repeat. One ORF encodes a bacteriophage-like DNA-dependent DNA polymerase (DdDp), which is similar to those encoded by protein-primed bacteriophages phi29 and PRD1. The other ORF encodes a T7-bacteriophage-like single-subunit DNA-dependent RNA polymerase (similar to nucleus-encoded mitochondrial RNA polymerases [Kempken, 1995b]). Many variations in size and internal organization exist, including plasmids that have a single large ORF encoding a DdDp or a DNA-dependent RNA polymerase, such as the pLm9 and pLm10 of *Leptosphaeria maculans* (Lim and Howlett, 1994). Single-ORF-encoding linear invertrons are often paired with a plasmid that encodes the other polymerase, suggesting that the two plasmids are dependent upon each other. In some instances, mitochondrial invertrons encode small ORFs of unknown identity that are typically found between the regions encoding the polymerases (Klassen and Meinhardt, 2007).

Circular DNA mt plasmids are covalently closed dsDNAs that replicate using a DdDp. They are approximately 5 kb in length and have a single long ORF that encodes a DdDp. The prototype is the LaBelle plasmid of *Neurospora intermedia* (Li and Nargang, 1993; Pande et al., 1989) which is 4.1 kb in length and encodes a 1,151-amino-acid ORF that shows similarity to virus-like family B DNA polymerases (Li and Nargang, 1993; Schulte and Lambowitz, 1991). The mechanism of replication is only partly understood; the LaBelle plasmid appears to be transcribed by the host mitochondrial RNA polymerase (Nargang et al., 1992) and following translation, the plasmid DdDp is thought to associate with the plasmid DNA and initiate DNA synthesis (Schulte and Lambowitz, 1991). The site of initiation of DNA synthesis and the nature of the primer are unknown. However, two-dimensional gel electrophoresis analysis of a circular DNA plasmid of *C. parasitica* (pCRY1) showed that the plasmid exists as discretely sized concatenated circular molecules with a varying amount of linear DNAs, suggesting that the plasmids replicate via a rolling-circle mechanism (Monteiro-Vitorello et al., 2000).

Plasmids classified as retroplasmids are dsDNAs that encode an RT or RNA-dependent DNA polymerase) and replicate via an RNA intermediate. Prototypic circular retroplasmids are the Mauriceville and Varkud plasmids of *Neurospora* species. These plasmids are covalently closed circular DNAs of approximately 3.6 kb and have 97% sequence identity, despite occurring in different species (Akins et al., 1988; Nargang et al., 1984). Both plasmids have a single large ORF that encodes a 725-amino-acid protein that has RT activity (Kuiper and Lambowitz, 1988; Kuiper et al., 1990). In vitro studies have demonstrated that the Mauriceville RT can initiate DNA synthesis without a primer (Wang and Lambowitz, 1993), which suggests that it may have a direct evolutionary relationship with RNA-dependent RNA polymerases. Strains containing Mauriceville or Varkud grow normally, but upon repeated vegetative transfer, they often become senescent. Presenescent cultures contain variant forms of the plasmids that contain cDNA copies of mitochondrial tRNAs (often along with other sequences) that appear to result from a template-switching event during plasmid replication (reviewed by Galligan and Kennell, 2007). Also included in this group is the VS plasmid of *N. intermedia*. It is a small (880-bp) dsDNA that does not encode any large ORFs. It appears to replicate via reverse transcription, using the RT encoded by the Varkud plasmid, and is thus considered to be a satellite plasmid (Kennell et al., 1995). Notably, the VS RNA transcript is a ribozyme and shows highly efficient RNA cleavage and ligation activity (Saville and Collins, 1990, 1991; Zamel et al., 2004). Only one other circular retroplasmid has been reported outside *Neurospora* species thus far: pThr1 of *Trichoderma harzianum* (Antal et al., 2002).

There are also linear dsDNA retroplasmids, whose prototypes are pFOXC2 and pFOXC3 of *Fusarium oxysporum*. These plasmids are 1.9-kb dsDNAs characterized by a "clothespin" structure having a terminal hairpin at one end and telomere-like repeats at the other containing a 5′-linked protein (Kistler et al., 1997; Walther and Kennell, 1999). The pFOXC plasmids contain a single large ORF that encodes an RT and are the first linear genetic elements shown to replicate via reverse transcription (Walther and Kennell, 1999). In vitro studies show that the pFOXC-RT has a loose specificity for primers, a characteristic that is likely to play a role in the addition of terminal repeats (Simpson et al., 2004). The existence of a plasmid that encodes an RT and has telomere-like ends suggests that it may have an evolutionary relationship to telomerase. A linear retroplasmid that has two terminal hairpins has been detected in *Rhizoctonia solani* (Katsura et al., 2001). Reviews of the replication cycle, phylogeny, and evolutionary significance of mitochondrial retroplasmids have been published recently (Galligan and Kennell, 2007; Hashiba and Nagasaka, 2007).

There are numerous reports of RNA elements being associated with mitochondria. Though they are formally classified as mitoviruses, they can also be considered plasmids based on their structural similarities to linear mt plasmids and lack of an extracellular form. The prototypical mitochondrial RNA plasmid is a 2.7-kb dsRNA found in *C. parasitica*, strain NB631 (Polashock and Hillman, 1994). It has a single ORF that encodes an RNA-dependent RNA polymerase believed to be involved in its replication. Other mitochondrial dsRNAs have been reported in *O. novo-ulmi* (Doherty et al., 2006; Hong et al., 1999), as has one in the basidiomycete *Helicobasidium mompa* (Osaki et al., 2005).

Surveys of *Neurospora* isolates show that more than one plasmid type can exist within an individual isolate, and in one case, seven different plasmid types were detected in a strain of *N. intermedia* (Yang and Griffiths, 1993b). Plasmids can recombine to form chimeric variants (even those of different types) indicating that they coexist within the same mitochondrion (Griffiths and Yang, 1995; Maas et al., 2007).

As expected of genetic elements that reside in mitochondria, mt plasmids are inherited maternally, yet they are also known to exploit alternative mechanisms of transmission, such as paternal inheritance (May and Taylor, 1989; Yang and Griffiths, 1993a) and transmission via anastomosis of fungal cells. Surveys of various filamentous fungal genera indicate that highly similar plasmids can be found in distantly related fungi, which implies that these elements are horizontally transmitted in nature (Arganoza et al., 1994; Griffiths, 1995). In laboratory experiments, mt plasmids are readily transmitted between vegetatively compatible strains (Debets et al., 1994) and have also been shown to overcome incompatibility barriers (Baidyaroy et al., 2000; Collins and Saville, 1990). Plasmid transfers between different species of *Neurospora* (Griffiths et al., 1990) and even between closely related fungal genera (Kempken, 1995a) have been reported. A thorough review of the inheritance mechanisms of fungal mt plasmids has been published (Griffiths, 1995).

MITOCHONDRIAL GENE EXPRESSION

Early studies involving *S. cerevisiae* showed that transcription of fungal mitochondrial genes utilizes a nucleus-encoded mitochondrial RNA polymerase, which is composed of two subunits, a catalytic core subunit (Masters et al., 1987) and a sigma-like specificity factor (Jang and Jaehning, 1991). The polymerase shows high similarity to single-subunit T3/T7-like RNA polymerase and appears to have replaced the multisubunit bacterial polymerase associated with the ancestral mitochondrial genome (Gray et al., 1998). The specificity factor binds to the core polymerase, creating a holoenzyme that recognizes the mitochondrial promoter, which in yeast is a nine-nucleotide consensus sequence. In *N. crassa*, the mitochondrial promoter consensus sequence differs slightly from yeast and is less conserved (Kennell and Lambowitz, 1989; Kubelik et al., 1990). Promoters are dispersed throughout the genome—the 78-kb mtDNA of *S. cerevisiae* contains 11 promoters on one strand and 1 promoter on the other

(Dieckmann and Staples, 1994)—and primary transcripts are most commonly multigenic and often terminate following a tRNA. Most mitochondrial genes appear to be constitutively expressed, and differential levels of RNA expression depend on the strength of the promoter. Interestingly, developmental regulation of several mitochondrial genes was reported in *N. crassa* during conidial germination; however, regulation appears to involve the recruitment of RNAs to the ribosome and is not dictated by the level of transcription (Bittner-Eddy et al., 1994).

Numerous processing events are required to generate mRNAs, several of which are transcript specific, while others, such as those that occur at the 3′ end of mRNA precursors, are performed by RNA endonucleases that cleave at conserved sequences (Min and Zassenhaus, 1993). The 5′ ends of tRNAs are cleaved by a mitochondrial RNase P (Miller and Martin, 1983; Morales et al., 1992), and 3′ ends are cleaved by a specific endonuclease (Chen and Martin, 1988) with CCA residues being added posttranscriptionally by a tRNA nucleotidyltransferase (Chen et al., 1992). Modification of nucleotides of tRNAs appear to occur in the nucleus, cytoplasm, and mitochondrion. In some lower fungi, the 5′ ends of certain tRNAs are edited (Forget et al., 2002; Laforest et al., 1997). As described above, RNA splicing of group I and group II introns involves intron-encoded maturase proteins as well as nucleus-encoded gene products. RNA turnover appears to play a role in the regulation of mitochondrial gene expression, with intergenic spacer regions cleaved from primary transcripts being rapidly turned over (Margossian and Butow, 1996). Some enzymes involved in RNA stability have been shown to be transcript specific (Dieckmann and Staples, 1994). While transcription, RNA processing, and degradation play a role in controlling the expression of certain mitochondrial genes, most regulation appears to occur during translation. In yeast, the synthesis of mitochondrial gene products is typically regulated by inner-membrane-bound translational activator proteins (Fox, 1996; Poyton and McEwen, 1996), and a similar example is known in *N. crassa* (Coffin et al., 1997). These proteins interact with both the ribosomes and the 5′ untranslated regions of mitochondrial mRNAs or help tether the synthesis of hydrophobic mitochondrial gene products to the inner membrane (Green-Willms et al., 1998). Many of these translational activators are mRNA specific, which may be necessary to provide individual control over gene products to coordinate the proper assembly of the large respiratory chain complexes.

IMPORT OF PROTEINS INTO MITOCHONDRIA

The large number of different proteins that are found in mitochondria (probably about 1,000 for fungi) and the limited coding capacity of mtDNA emphasize the fact that most mitochondrial proteins are encoded by nuclear genes, translated in the cytosol, and imported into the organelle. Incoming proteins must also be sorted to the correct mitochondrial compartment for proper function. These processes are achieved by multisubunit translocases housed within the MOM and MIM. Although *S. cerevisiae* has been the dominant organism in the field since the mid-1990s, several important contributions have come from studies with *N. crassa*. Here we attempt to define the basic import process and briefly summarize the literature while pointing out contributions that have involved filamentous fungi—almost entirely *N. crassa*.

The term "precursor" or "preprotein" is used for mitochondrial proteins before they reach their final destination and conformation within the mitochondrion. The precursors of many mitochondrial proteins carry an N-terminal extension referred to as a "targeting sequence" or a "presequence," which is ultimately removed from the protein. Presequences usually contain hydroxylated and positively charged amino acids, lack negatively charged amino acids, and are capable of forming an amphipathic helix that presents the positively charged residues on one face and hydrophobic residues on the other (von Heijne, 1996). For precursors without presequences, the mitochondrial targeting information is found within the mature protein sequence.

Early studies using *N. crassa* showed that translation of precursor proteins on cytosolic ribosomes was not directly coupled to their import into mitochondria. Rather, they are released from ribosomes into the cytosol and interact with the mitochondrial import apparatus in a posttranslational fashion (Hallermayer et al., 1977; Harmey et al., 1977). However, there may also be certain proteins or conditions that favor cotranslational import (Ahmed et al., 2006; Lithgow, 2000; Verner, 1993).

Complexes Used for Import of Proteins into Mitochondria

The import complexes found in both the MOM and the MIM are shown and defined in Fig. 3 and its legend. The nomenclature for import complexes and their components was unified in 1996 (Pfanner et al., 1996). Complexes are referred to in capital letters (e.g., TOM). Proteins of the complexes use the same letters, with only the first letter capitalized (e.g., Tom). Numbers following the letters designate the molecular mass in kilodaltons of a specific protein (e.g., Tom40). A somewhat confused nomenclature has arisen for those complexes and subunits discovered since the unification. This is most evident for the SAM or TOB complex (see below), and the alternate names for its components are listed in Table 2. In this chapter, we use the SAM complex terminology. Another nomenclature discrepancy has developed with respect to the import motor associated with the TIM23 complex. One view sees the motor as a complex that is separate from the TIM23 complex and refers to it as the PAM (preprotein translocase-associated motor) complex. A different view is that the motor is simply part of the TIM23 complex. Here we refer to the PAM complex, but we also give the most common alternate names for subunits of the complex.

The TOM complex

Preproteins are recognized and bound on the outer surface of the MOM (cytosolic side) by specific receptors in the TOM complex and are subsequently routed through the TOM complex translocation channel or pore. Precursors are kept in an import-competent state through interaction with chaperones and other factors in the cytosol (reviewed by Neupert, 1997, and Neupert and Herrmann, 2007). The TOM complex of the MOM is the entry point for almost all mitochondrial proteins into the organelle. The holo-TOM complex, which can be isolated following solubilization of mitochondria in the mild detergent digitonin, consists of seven subunits (Fig. 3). Stronger detergents remove Tom70 and Tom20 and give rise to the core TOM complex (Ahting et al., 1999). All the subunits of the TOM complex are held in the membrane by a single membrane-spanning domain with the exception of Tom40. Computer predictions and circular dichroism studies (Ahting et al., 2001; Becker et al., 2005) have shown that Tom40 exists as a β-barrel in the MOM.

The TOM complex from *N. crassa* has been visualized by electron tomography (Ahting et al., 1999, 2001;

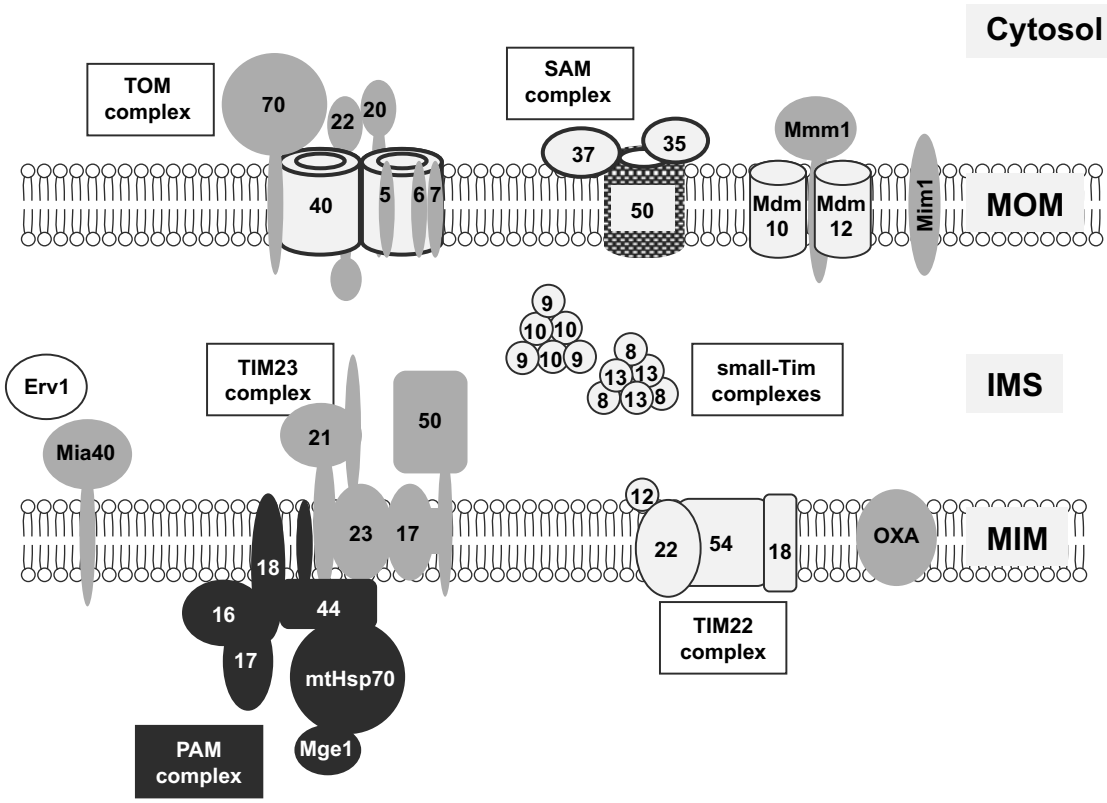

FIGURE 3 Complexes and proteins involved in import of proteins into mitochondria. Subunits of each complex are shown with a number representing their molecular mass. The TOM complex (translocase of the outer mitochondrial membrane) is the entry point for the majority of mitochondrial proteins. The SAM complex (sorting and assembly machinery) is also known as the TOB (topogenesis of β-barrel proteins) complex (see Table 2). The SAM complex is responsible for the insertion of β-barrel proteins into the outer membrane following their import through the TOM complex into the IMS. Further assembly of β-barrel proteins requires the action of Mdm12 and Mmm1. Tom40 additionally requires the action of Mdm10 and Mim1. The two TIM complexes (translocases of the inner mitochondrial membrane) were named for the Tim22 and Tim23 proteins originally identified as components of the respective complex. Precursors of the carrier family (such as the ATP/ADP carrier) and a few other proteins with an even number of membrane-spanning domains and no cleavable presequence are inserted into the MIM by the TIM22 complex. Precursors with cleavable targeting signals are recognized as substrates by the TIM23 complex and translocated into the matrix. However, some TIM23 substrates contain stop-transfer signals that halt movement into the matrix and result in partitioning into the MIM. The PAM complex (presequence translocase associated motor) binds the N terminus of presequence-containing preproteins as they enter the matrix. Through cycles of interaction with mtHsp70, the precursor is drawn into the matrix. Two complexes composed of six small Tim protein subunits, either three subunits of Tim8 plus three of Tim13 or three of Tim9 plus three of Tim10, exist in the IMS. These complexes chaperone precursors en route to either the SAM complex or the TIM22 complex through the IMS. The OXA (cytochrome oxidase assembly) complex "exports" a small class of matrix targeted precursors back into the MIM from the matrix. It is also involved in the insertion of mitochondrial translation products into the MIM. The Mia40 and Erv1 proteins form a disulfide relay system that is responsible for trapping a class of small precursors with a twin cysteine motif into the IMS. Further details are given in the text.

Künkele et al., 1998a). Purified preparations of the holo-TOM complex were seen to contain structures with two or three pores, while the core complex contained mostly two-pored structures (Fig. 4). When purified from mitochondria by using the detergent octylglucoside, the complex contained only Tom40 and gave images containing only one pore (Ahting et al., 2001). Comparison of the *S. cerevisiae* wild-type complex with the complex isolated from strains lacking Tom20 revealed that the presence of Tom20 correlated with the existence of the three-pored structure (Model et al., 2002). The pores within the TOM complex structure have been estimated to be about 20 to 26 Å in diameter by both electron microscopic analysis and size exclusion studies (Ahting et al., 1999, 2001; Hill et al., 1998; Künkele et al., 1998a; Schwartz and Matouschek, 1999).

Tom40 is the major component of the TOM complex pore (Hill et al., 1998; Künkele et al., 1998a, 1998b). The gating characteristics of the TOM core complex and purified Tom40 from *N. crassa* have been compared by electrophysiological analysis. The presence of the non-Tom40 components of the core complex reduced the energy needed for transitions between different conductance states of the pore. Examination of core complex isolated from mutant strains lacking one of Tom5, Tom6, or Tom7 showed no

TABLE 2 Alternate names for the SAM complex and its components

Name used in this chapter	Synonyms
SAM complex (sorting and assembly machinery)	TOB complex (topogenesis of β-barrel proteins)
Sam50	Tob55, Omp85
Sam37	Mas37, Tom37, Metaxin 1 (mammalian)
Sam35	Tob38, Tom38, Metaxin2 (mammalian)

differences from the wild type, suggesting that Tom22 and/or some combination of the smaller Tom components is required for the effect (Poynor et al., 2008).

The cytoplasmic domains of Tom70, Tom22, and Tom20 act as receptors for mitochondrial precursor proteins. The C-terminal domain of Tom20 contains a series of alpha helices that form a groove for binding presequences through hydrophobic interactions via a dynamic mechanism that allows recognition of a wide range of mitochondrial presequences (Abe et al., 2000; Saitoh et al., 2007). Tom22 is required for maintaining TOM complex organization (van Wilpe et al., 1999) and has been shown to interact with Tom20, where it assists with the binding of precursors (Mayer et al., 1995; Yamano et al., 2008) and promoting their passage from the receptor to the translocation pore (Kiebler et al., 1993). Tom70 is the usual receptor for hydrophobic precursors with multiple membrane-spanning domains and internal targeting signals that are destined for the MIM (Ryan et al., 1999; Söllner et al., 1990; Young et al., 2003). Such proteins include members of the carrier family like the ATP/ADP carrier. Though the precursors that interact with Tom70 are fairly specialized, when Tom70 is absent, Tom20 is able to fulfill its function.

There appears to be some variability between yeast and *N. crassa* with respect to the function of the three small components of the TOM complex. In yeast, Tom5 was found to play an important role in the transfer of precursors from the receptors to the translocation channel (Dietmeier et al., 1997), but no effect on import was seen in *N. crassa* strains lacking the protein (Schmitt et al., 2005). In yeast, Tom6 is thought to promote the association of receptors with Tom40, while Tom7 has the opposite function and decreases stability of receptor/Tom40 interactions (Alconada et al., 1995; Dekker et al., 1998; Hönlinger et al., 1996). In *N. crassa*, both proteins increase complex stability (Sherman et al., 2005). Tom7 and the IMS domain of Tom22 may form part of a site for binding precursors on the inner side of the TOM complex (Esaki et al., 2004).

The SAM Complex and Assembly of β-Barrel Proteins

The SAM complex (Fig. 3; Table 2) is responsible for the insertion of β-barrel proteins into the MOM. The currently known β-barrel proteins of the MOM are Tom40, porin, Sam50, Mdm10, and Mmm2. The SAM complex contains three core components Sam50, Sam35, and Sam37 (Kutik et al., 2007; Milenkovic et al., 2004; Neupert and Herrmann, 2007; Waizenegger et al., 2004). Sam50, predicted to be a β-barrel protein itself, is the core of the complex and forms what appears to be an insertion channel (Paschen et al., 2003). In *N. crassa*, three different versions of Sam50 occur as the result of alternative splicing (Hoppins et al., 2007). In yeast, Sam35 and Sam37 are peripheral membrane proteins on the cytosolic side of the MOM, but the mammalian homologue of Sam37 (metaxin 1) appears to be anchored in the MOM by a single membrane-spanning domain (Abdul et al., 2000; Armstrong et al., 1997).

The assembly of β-barrel precursors has been intensively studied in both yeast and *N. crassa* with a focus on the assembly of the Tom40 precursor (Model et al., 2001; Rapaport and Neupert, 1999; Taylor et al., 2003). As shown in Fig. 5, a newly synthesized 40-kDa precursor binds to receptors on the cytosolic side of the TOM complex and is subsequently translocated through the pore of the complex. Studies involving both *N. crassa* and yeast demonstrated that as the precursor emerges from the TOM complex to the IMS side of the outer membrane, it associates with the Tim8/Tim13 or Tim9/Tim10 complex (Hoppins and Nargang, 2004; Wiedemann et al., 2004). Presumably these complexes act as chaperones to guide the precursor to the SAM complex. The incoming Tom40 precursor then associates with the SAM complex, resulting in the formation of a 250-kDa intermediate known as assembly intermediate I. This association is transient, and the SAM complex inserts Tom40 into the outer membrane. Insertion of β-barrel proteins into the MOM is dependent on a specific sorting signal that occurs in the last predicted β-strand of the proteins. Interestingly, the receptor for the signal is Sam35, which resides on the cytosolic side of the MOM. It is thought that Sam35 is closely associated with the

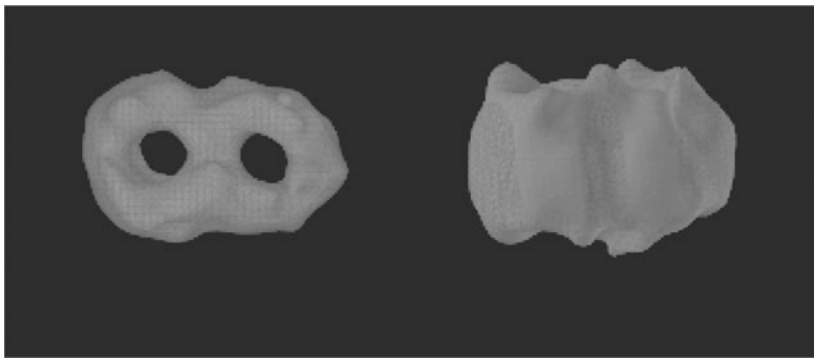

FIGURE 4 Electron tomographic image of the purified *N. crassa* TOM core complex. The stain-filled pits are assumed to be pores through which preproteins cross the MOM. (From Ahting et al., 1999.)

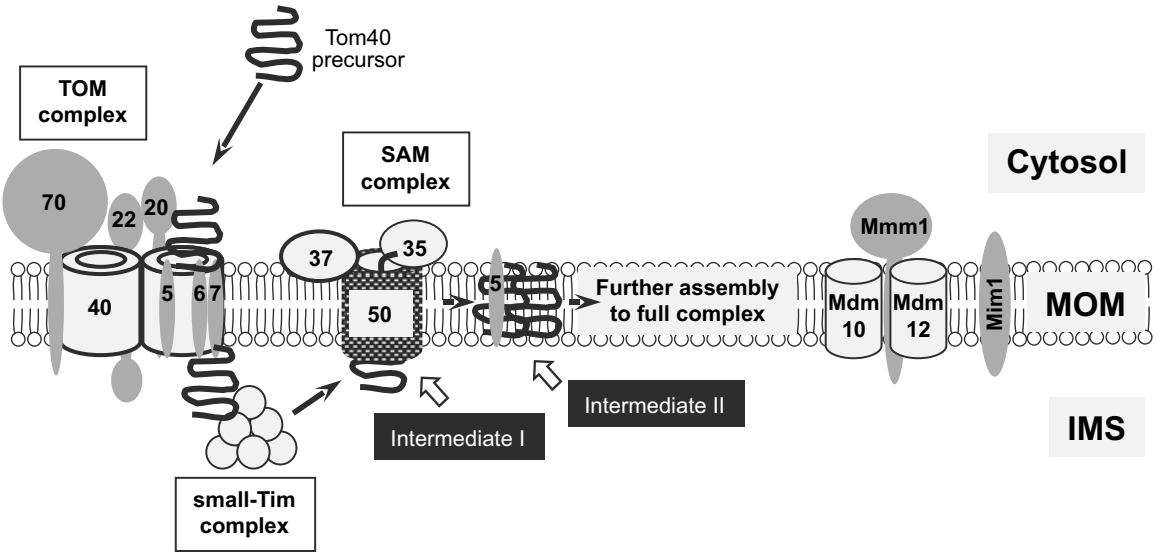

FIGURE 5 Steps in assembly of Tom40 into the TOM complex. Following synthesis in the cytosol, a Tom40 precursor is recognized by the TOM complex and imported into the IMS. The precursor then interacts with one of the small-Tim complexes that chaperone the precursor to the SAM complex. The precursor interacts with the SAM complex and forms assembly intermediate I. The SAM complex inserts the precursor into the membrane, where it associates with a preexisting Tom40 molecule and a molecule of Tom5 to give assembly intermediate II. Further assembly gives the fully formed TOM complex. The Mdm10, Mdm12, Mmm1, and Mim1 proteins are also required for efficient assembly of Tom40 at steps following interaction with the SAM complex.

membrane domains of Sam50 and that the β-signal extends through the SAM complex to interact with Sam35. Interaction of the β-signal with Sam35 results in a change in the conductance and increase in size of the SAM complex channel so that several β-strands of the incoming precursor could insert at the same time. These data suggest that the insertion of β-barrel proteins into the channel occurs with the precursor in at least a partially folded conformation followed by lateral release from the channel into the membrane (Kutik et al., 2008). The steps up to and including membrane insertion of Tom40 serve as a general model for the assembly of all β-barrel proteins into the MOM (Kutik et al., 2008; Paschen et al., 2003). Following insertion, there are different pathways for further assembly depending on the particular β-barrel protein. For Tom40, insertion is followed by association with another Tom40 subunit and a Tom5 subunit to form a 100-kDa intermediate known as assembly intermediate II. Further interactions with additional TOM complex subunits give rise to the fully assembled 400-kDa TOM core complex. However, additional proteins are needed for efficient assembly of Tom40 and other β-barrel proteins.

Three proteins originally described for their roles in mitochondrial distribution and morphology (mdm) or maintenance of mitochondrial morphology (mmm) have now been shown to be involved in β-barrel assembly pathways. The Mdm10, Mdm12, and Mmm1 proteins were known to exist in a complex, and the absence of any one of them resulted in altered mitochondrial morphology in yeast (Boldogh et al., 2003). Altered mitochondrial morphology was also shown for *mmm1* mutants in *N. crassa* (Prokisch et al., 2000). However, Mdm12 and Mmm1 have now been shown to be required for efficient assembly of all β-barrel proteins (Meisinger et al., 2007), while Mdm10 is specifically required for efficient assembly of Tom40 into the TOM complex (Meisinger et al., 2004). Since β-barrel precursors in mutant strains lacking these proteins are able to form interactions with the SAM complex, it is thought that the requirement for these proteins occurs at later steps in the assembly pathway. It seems likely that the defects in mitochondrial morphology associated with the loss of any of these three proteins are a secondary effect that is revealed as a result of interruptions in the β-barrel assembly pathway (Meisinger et al., 2007). Another protein named Mim1 (or Tom13) also plays a role in the assembly of Tom40 into the TOM complex at a point after association with the SAM complex (Becker et al., 2008; Ishikawa et al., 2004; Waizenegger et al., 2005).

Import Complexes Found in, or Associated with, the MIM

The vast majority of the work on the TIM23, TIM22, PAM, and OXA complexes as well as the IMS oxidative folding pathway has been done using *S. cerevisiae* with few contributions coming from filamentous fungi. Nonetheless, for completeness, the function of these complexes is summarized here briefly. The work is described in several recent general reviews (Bohnert et al., 2007; Bolender et al., 2008; Gakh et al., 2002; Koehler, 2004; Kutik et al., 2007; Milenkovic et al., 2007; Neupert and Herrmann, 2007) and in more specific reviews given in the appropriate sections. References to original work are cited only for recent work not covered in reviews or for examples of studies that have involved filamentous fungi.

The TIM23 Complex and the PAM Complex

Preproteins destined for the matrix emerge on the IMS side of the TOM complex as they cross the MOM. Further

translocation is initiated by the TIM23 complex (Fig. 3), which contains Tim50, Tim23, Tim21, and Tim17. Tim50 interacts with the incoming precursor and passes it to the translocation channel. The channel appears to be formed by Tim23, but Tim17 is involved in channel gating (Martinez-Caballero et al., 2007). In a membrane potential-dependent reaction, the positively charged presequence of the preprotein is moved through the Tim23 channel (Krayl et al., 2007). Tim21 has been proposed to connect the TOM and TIM23 complexes and may assist in releasing the precursor from the TOM complex. Movement of the remaining portion of the preprotein through the channel requires the PAM complex (also known as the motor segment of the TIM23 complex). Tim50 plays another important role by interacting with Tim23 when no preproteins are present to prevent leakage of protons from the IMS to the matrix through the Tim23 pore.

The PAM complex (Fig. 3) contains Tim44, Pam18 (also called Tim14), Pam16 (also called Tim16), Pam17 mtHsp70, and Mge1. Tim44 binds to the matrix side of the TIM23 complex and recruits the ATP bound form of the mtHsp70 chaperone to the site (Schiller et al., 2008). The incoming precursor is then bound by mtHsp70-ATP resulting in hydrolysis of the ATP molecule and the formation of an mtHsp70-ADP complex that is tightly bound to the precursor. This complex is released from Tim44, allowing a new mtHsp70-ATP complex to bind. The Mge1 protein associates with the mtHsp70-ADP complex and stimulates release of the precursor and ADP from mtHsp70. This cycle is repeated until the precursor is translocated into the matrix. Hydrolysis of ATP by mtHsp70 is regulated by Pam18 (Tim14) and Pam16 (Tim16). Pam17 is thought to stabilize an association between the Pam16 (Tim16) and Pam18 (Tim14) subunits. The presequence of incoming precursor proteins is removed upon its entrance into the matrix by the matrix processing peptidase. Tam41 (also called Mmp37), though not a component of the complex, was reported to be required to maintain the integrity of the TIM23 complex.

There is debate as to how the energy gained from hydrolysis of ATP by mtHsp70 is actually used to drive translocation into the matrix (Neupert and Brunner, 2002; Pfanner and Truscott, 2002). The ratchet model suggests that spontaneous unfolding of the portion of the precursor still on the cytosolic side of the MOM would allow sliding of the precursor back and forth within the TOM and TIM23 pores. However, since mtHsp70 binds to the precursor as it enters the matrix, this would prevent backsliding and impart a net vectorial movement into the matrix as the more-C-terminal regions of the precursor are progressively bound by mtHsp70. The power stroke or active-pulling model suggests that mtHsp70 exerts a pulling force on the precursor via conformational changes that occur when ATP is hydrolyzed. The entropic pulling model suggests that entropy loss as the result of excluded volume effects upon the binding of mtHsp70 to incoming precursors drives movement (De Los Rios et al., 2006).

Several proteins destined for the IMS or the MIM also use the TIM23 pathway to reach their destinations. In these cases, the presequence is followed by a stop-transfer signal that arrests translocation of the precursor in the TIM23 complex and results in its lateral insertion into the MIM. The presequence is also removed from these preproteins by matrix processing peptidase resulting in an integral MIM protein. Some of these proteins undergo further cleavage by an inner membrane peptidase that removes the stop-transfer signal from the mature peptide (Gakh et al., 2002). This results in a final IMS location for the imported protein. It has been suggested that a TIM23 complex associated with the PAM complex, but without the Tim21 protein, accomplishes translocation into the matrix, whereas the membrane portion of the TIM23 complex with Tim21, but without the PAM complex, inserts precursors into the inner membrane (Chacinska et al., 2005; van der Laan et al., 2007). However, other studies concluded that the TIM23 membrane complex and the PAM complex were always associated, regardless of the type of preprotein being imported. In this study, different conformations of the entire complex were detected depending on the nature of the precursor being imported (Popov-Celeketic et al., 2008).

Oxidative Folding Drives Import of Small IMS Proteins

Many small proteins destined for the IMS, including the small Tim proteins (Fig. 3), contain characteristic twin cysteine motifs and use an oxidative-folding, disulfide relay system to drive their import (reviewed by Hell, 2008, and Stojanovski et al., 2008). When these proteins enter the IMS from the TOM complex, they interact with an oxidized form of the Mia40 protein. Mia40 catalyzes the formation of intramolecular disulfide bonds within the proteins, converting them to an oxidized folded state that conformationally traps them in the IMS. Mia40 is reduced as a result of the reaction, and the Erv1 protein reoxidizes Mia40 so that its catalytic function is restored. Reoxidation of Erv1 is accomplished by passage of its electrons to molecular oxygen or oxidized cytochrome *c*.

The TIM22 Complex

The TIM22 complex (Fig. 3) imports the carrier proteins (such as the ATP/ADP carrier) as well as TIM complex subunits with multiple membrane-spanning domains to the MIM (reviewed by Jensen and Dunn, 2002, and Rehling et al., 2004). These hydrophobic proteins are chaperoned across the IMS by the small Tim protein complexes (Tim8-Tim13 and Tim9-Tim10) and bound by a Tim9-Tim10-Tim12 complex associated with the TIM22 complex on the IMS side of the MIM. Tim22 is the pore-forming component of the complex and mediates the insertion of the precursor protein into the MIM in the presence of membrane potential. Tim54 and Tim18 are thought to be involved in TIM22 complex assembly. A role for Tim54 in the assembly of the Yme1p protease complex of the MIM has also been demonstrated (Hwang et al., 2007).

OXA Translocase

A number of nucleus-encoded proteins are known to be imported into the matrix via the TIM23 pathway but then "exported" back into the MIM. For many of these proteins the insertion is catalyzed by the Oxa1 translocase (Fig. 3) of the MIM (reviewed by Bonnefoy et al., 2008, and Stuart, 2002). Oxa1 is also involved in the insertion of mitochondrially translated proteins into the MIM. In *S. cerevisiae* it has been shown that mitochondrial ribosomes interact with Oxa1 to facilitate insertion of proteins in a cotranslational manner. Work with *N. crassa* demonstrated that the protein probably exists as a homotetrameric complex in the MIM (Nargang et al., 2002). Oxa1 is essential for viability in both *N. crassa* (Nargang et al., 2002) and *P. anserina* (Sellem et al., 2005).

COORDINATION OF NUCLEAR AND MITOCHONDRIAL GENOMES

Since proper formation of mitochondria requires the expression of a small number of genes within the organelle and a large number of genes in the nucleus, it has long been thought that there must be systems of communication to ensure that mitochondrial biogenesis occurs in a regulated fashion. The term "retrograde regulation" has been used to describe the signaling from mitochondria that influences the expression of nuclear genes. Although some of the earliest reports on mitochondrial to nuclear signaling were from work with *N. crassa* (Barath and Kuntzel, 1972a, 1972b), retrograde signaling has been most intensively studied in *S. cerevisiae*, where many genes functioning in various retrograde pathways have been described (reviewed by Butow and Avadhani, 2004).

AOX as a Model for Retrograde Regulation

Alternative oxidase (AOX) is a nucleus-encoded protein that represents an excellent model for retrograde regulation because its expression is often influenced by the functional status of the standard cytochrome-mediated electron transport chain (cmETC) (Fig. 1). AOX is found in several species of fungi and yeasts (but not *S. cerevisiae*), protists, bacteria, and animals as well as in all green plants (Chaudhuri et al., 2006; Finnegan et al., 2004; Joseph-Horne et al., 2001; McDonald and Vanlerberghe, 2006; Rhoads and Vanlerberghe, 2004). AOX transfers electrons directly from ubiquinol to oxygen. When both the AOX and cmETC pathways are fully or partially functional, a branched pathway is formed (Fig. 1). The AOX is not sensitive to the same inhibitors that act on the cytochrome-mediated electron transport chain such as cyanide (inhibits complex IV) or antimycin A (inhibits complex III). Thus, AOX is often described as the cyanide-insensitive or cyanide-resistant respiration pathway. AOX is inhibited by compounds such as salicylhydroxamic acid and *n*-propylgallate.

Electron transfer via the AOX pathway bypasses the last two proton-pumping sites of the cmETC. Thus, the energy that normally would have been used to create a proton gradient and synthesize ATP is lost as heat. This makes the alternative pathway inefficient in terms of energy conservation. However, under conditions where mutations or inhibitors have blocked one of the later steps of the standard respiratory pathway, recycling of electron carriers and ATP production via proton pumping at complex I can continue via the AOX (Fig. 1). Thus, one function of the AOX may be to provide an escape mechanism from inhibitors of the latter portion of the cmETC so that the negative effects of naturally occurring molecules like sulfide and cyanide might be avoided (Joseph-Horne et al., 2001; McDonald and Vanlerberghe, 2004).

AOX has been observed in many filamentous fungi and has been studied in detail with respect to its expression in a few species. In 1953, cyanide-insensitive respiration in the cytochrome aa_3 and *b* deficient (*poky*) mutant of *N. crassa* was reported (Tissieres et al., 1953). Subsequently it was shown that this alternative pathway of respiration was present in the mitochondria of *poky* and that the AOX and cmETC form a branched pathway (Lambowitz et al., 1972). It was also demonstrated that the *N. crassa* enzyme could be induced not only by growth in the presence of the cmETC inhibitors cyanide and antimycin A, but also by chloramphenicol, ethidium bromide, and oligomycin (Bertrand et al., 1983; Edwards et al., 1974, 1976; Lambowitz and Slayman, 1971). Chloramphenicol inhibits mitochondrial protein synthesis and results in a reduction of complexes I, III, and IV, which contain mitochondrially encoded components. Ethidium bromide inhibits mitochondrial transcription so that it also reduces the level of complexes containing components encoded by mtDNA. Induction by oligomycin is likely due to inhibition of electron transport that occurs with the buildup of the proton gradient that cannot be dissipated due to inhibition of ATP synthase. In addition to *poky*, other mutations affecting the cmETC also resulted in AOX expression (Bertrand et al., 1983; Li et al., 1996) as did growth in copper-deficient medium (Schwab, 1973), since Cu is a constituent of complex IV. Taken together, these observations suggest that any condition resulting in a reduction of the function of the cmETC induces AOX in *N. crassa*.

The regulation of AOX expression has been of considerable interest since its discovery. Transcription of nuclear DNA was shown to be required for the appearance of AOX activity (Edwards and Unger, 1978). Nuclear run-on assays and quantitative PCR have shown that there is a low level of constitutive transcription of the *N. crassa* gene (Chae et al., 2007a; Tanton et al., 2003). However, the near-total absence of cyanide-insensitive respiration or AOX protein in uninduced cells suggests a mechanism of posttranscriptional control that prevents expression of the mRNA. Induction of the AOX occurs quite rapidly. A 1-h exposure of growing cells to antimycin A increased the rate of AOX transcription about sevenfold (Tanton et al., 2003). Longer exposure to antimycin A led to about a 17-fold increase in transcript levels (Chae et al., 2007a). AOX activity was detected after about 30 min following addition of chloramphenicol or ethidium bromide to growing cultures (Edwards et al., 1974, 1976).

N. crassa mutants unable to express AOX were originally isolated by using selection schemes centered on the inability of strains lacking AOX to grow in the presence of antimycin A (Bertrand et al., 1983; Edwards et al., 1976). These screens led to the identification of two genes required for AOX production, known as *aod-1* and *aod-2* (for alternative oxidase deficient). Using a monoclonal antibody developed against the *Sauromatum guttatum* AOX protein, it was shown that most *aod-1* mutants still produced AOX protein, whereas *aod-2* mutants did not. This led to the hypothesis that *aod-1* was the structural gene for AOX while *aod-2* encoded a factor required for AOX expression (Bertrand et al., 1983; Lambowitz et al., 1989). When the *N. crassa aod-1* gene was cloned, it was shown that the level of the *aod-1* message was severely reduced in an *aod-2* mutant, supporting the hypothesis that *aod-2* was involved in controlling the transcription or stability of the *aod-1* mRNA (Li et al., 1996). A second AOX structural gene (*aod-3*) was discovered in *N. crassa* by homology searches (Tanton et al., 2003) after the completion of the *N. crassa* genome sequence (Galagan et al., 2003). However, conditions leading to expression of *aod-3* have not been identified, and the gene clearly plays no role under conditions where *aod-1* function is required, since strains containing mutations affecting the *aod-1* gene do not grow in the presence of antimycin A (Descheneau et al., 2005; Tanton et al., 2003).

The cloning of *aod-1* allowed the development of a reporter system screen to select specifically for regulatory mutants unable to induce *aod-1* expression. Use of this system led to the discovery of four additional genes (*aod-4*, *aod-5*, *aod-6*, and *aod-7*) required for *aod-1* expression (Descheneau

et al., 2005). Thus, a total of five putative AOX regulatory loci may exist, and since the mutant screen was not saturated, there may be a very complex pathway(s) controlling production of the enzyme. Recently, two of the potential regulatory genes (*aod-2* and *aod-5*) were identified as zinc cluster transcription factors necessary for the expression of *aod-1*. Electrophoretic mobility shift assays demonstrated that the two factors bind as a heterodimer to the AOX induction motif (AIM) found about 170 bases upstream from the start of the structural gene coding sequence (Chae et al., 2007a).

The AIM sequence is typical of those recognized by zinc cluster transcription factors (MacPherson et al., 2006) and contains two directly repeated CGG triplets separated by 7 bp (Chae et al., 2007b). The AIM was found to be conserved in other species of the order Sordariales but was not detected in more distantly related fungi known to express AOX, so that the mechanism for regulation of expression of AOX may vary among filamentous fungi and other organisms (Chae et al., 2007a). The current challenge in *N. crassa* is to identify the signal(s) that activates the AOX transcription factors and to determine the identity of the other potential members of the signal transduction pathway.

In *Magnoporthe grisea* the alternative pathway is induced following exposure to the fungicide SSF-126 [(E)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl) acetamide], a complex III inhibitor (Mizutani et al., 1995). Nuclear run-on assays showed that the AOX was also transcribed constitutively in this species (Yukioka et al., 1998a), although exposure of cells to SSF-126 increased the rate of transcription. Since SSF-126 was shown to result in the generation of reactive oxygen species (ROS), and since hydrogen peroxide also induced transcription of AOX, it was suggested that ROS may act as an AOX induction signal in *M. grisea*. The constitutively expressed transcript was thought to be actively degraded by a factor sensitive to cycloheximide. AOX-deficient mutants affecting the structural gene have been isolated in *M. grisea*, and potential regulatory elements in the promoter region have been identified, but these have not been directly tested for a role in production of the transcript (Yukioka et al., 1998b).

M. grisea is a plant pathogen that causes rice blast disease. Quinol oxidation inhibitors, acting at complex III of the cmETC, are a major class of fungicides used to combat *M. grisea* and other fungal plant pathogens. Unexpectedly, the fungal AOX does not provide an escape from the action of these inhibitors, except possibly in the later stages of infection. It is thought that plant antioxidants released during infection may reduce ROS, which seem to be required for AOX induction in these pathogenic species (reviewed by Fernandez-Ortuno et al., 2008).

Candida albicans contains two AOX genes, named *AOX1a* and *AOX1b*. Although the *AOX1a* transcript could not be detected on Northern blots, a reporter gene fused to its promoter showed constitutive expression. Expression of *AOX1b* was dependent on the growth phase and could be induced by cyanide, antimycin A, paraquat (*N*,*N*′-dimethyl-4-4′-bipyridinium dichloride), hydrogen peroxide, and menadione. Interestingly, although an *aox1a* mutant strain was capable of inducing AOX (presumably from *AOX1b*) following a short treatment with antimycin A, growth of the strain in the presence of potassium cyanide or antimycin A was severely reduced. There was virtually no growth of an *aox1b* mutant or a double *aox1a aox1b* mutant in the presence of these inhibitors. It was also shown that in cells deleted for the CaSLN1 gene, which encodes a histidine kinase, AOX activity and protein levels were slightly reduced under normal conditions and slightly increased in the presence of antimycin A. This suggested that the histidine kinase may be involved in the regulation of AOX expression in this species (Huh and Kang, 2001). It has recently been reported that erythroascorbic acid stimulates AOX production in *C. albicans* as well as having a general stimulating effect on respiration (Huh et al., 2008). The mechanisms involved are not yet understood. The activity of MnSOD has also been found to influence *C. albicans* AOX expression, probably via its effect on mitochondrial ROS levels (Hwang et al., 2003). Finally, it has been suggested that AOX plays a role in the pH-controlled transition between yeast and hyphal forms of *C. albicans* (Konno et al., 2006).

AOX in *P. anserina* and Its Relationship to Senescence

As described below, *P. anserina* is unusual among filamentous fungi in that it has a limited life span and undergoes senescence after a strain-specific period of growth. Senescence is associated with rearrangements in the mtDNA that lead to a gradual decline in mitochondrial function in this obligate aerobic organism. As in other organisms, *P. anserina* expresses AOX in response to impairment of the standard electron transport chain by mutations (Borghouts et al., 2001; Dufour et al., 2000). Interestingly, the decline in electron transport caused by the effects of the natural senescence mechanism on the cmETC complexes does not result in the induction of AOX (Borghouts et al., 2001). Two possible explanations for this observation were suggested. First, the organism may have a mechanism that prevents AOX induction during senescence, since the degeneration of mtDNA that occurs results in the loss of mitochondrial genes, including those for complex I. Thus, the AOX activity would have no purpose during senescence, because the ability to create a proton gradient and synthesize ATP via complex I would not exist even if the AOX were in place. Second, it was shown that transcription of *P. anserina* AOX was reduced when the growth medium was supplemented with copper (Borghouts et al., 2001; Stumpferl et al., 2004). Since the cytoplasmic copper concentration in cells undergoing senescence is thought to increase (Averbeck et al., 2001; Borghouts et al., 2001, 2002), this may have a negative effect on AOX expression.

There appears to be a low level of constitutive transcription of the AOX gene in wild-type strains, which can be increased severalfold in mutants affecting the cmETC (Borghouts et al., 2001; Dufour et al., 2000). However, different results have been reported as to whether or not the protein is expressed in noninduced cultures (Borghouts et al., 2001; Gredilla et al., 2006; Lorin et al., 2001; Sellem et al., 2005; Stumpferl et al., 2004). Unlike what has been seen in many organisms, transcript levels of AOX were not induced by ROS in *P. anserina*, as judged by examination of AOX following growth in the presence of hydrogen peroxide or paraquat (Borghouts et al., 2001). In fact, a reduction in transcript levels was seen. It was not known if transcript production was repressed or if these compounds decreased transcript stability.

Long-lived strains of *P. anserina* that carry mutations affecting the cmETC have been isolated (Belcour and Vierny, 1982; Schulte et al., 1988). As a result of the cmETC respiratory defects, AOX is induced in such strains. For example, studies with a mutant lacking a functional complex IV of the cmETC due to a disruption in the nucleus-encoded

COX5 subunit of the complex showed that AOX was induced, ROS production was reduced, mtDNA rearrangements were eliminated, and life span was dramatically increased (Dufour et al., 2000). Since it is thought that ROS may play a role in inducing senescence, and since an inverse correlation between ROS and AOX levels was observed in plants (Maxwell et al., 1999; Purvis, 1997; Wagner and Moore, 1997), it was conceivable that respiration via AOX in *P. anserina* could have accounted for all the phenotypic properties of the mutant. However, it has now been shown that there is no direct link between AOX levels, ROS, and life span in *P. anserina*, since overexpression of AOX in the *cox5* mutant strain severely decreased the life span and returned ROS production to wild-type levels (Lorin et al., 2001). Similar observations were made when AOX was overexpressed in a mutant lacking cytochrome c_1 and complex III function (Sellem et al., 2007). In addition, inactivation of the gene encoding AOX did not affect the life span of an *oxa1* mutant which was characterized by decreased levels of complexes I and IV, strongly induced AOX levels, and an increased life span (Sellem et al., 2005).

Other investigations of AOX and life span in *P. anserina* have focused on copper metabolism. The effects of cellular copper depletion were examined in a mutant affecting a transcription factor called *GRISEA*, which is required for expression of a copper permease (Borghouts et al., 2001). These mutant cells were also found to have an increased life span, and this was related to several events in the cell. Complex IV of the standard electron transport chain was reduced about fivefold in the mutant due to its use of copper as a cofactor. The *SOD2* gene, encoding mitochondrial Mn-SOD2, was not expressed because it requires *GRISEA* for transcription. Finally, AOX was induced in these cultures most likely because of the reduction in complex IV activity. It was suggested that ROS levels would be decreased due to respiration via the AOX and that this would result in a longer life span (Borghouts et al., 2001; Gredilla et al., 2006).

The following model has been suggested as an explanation for the relationship between respiratory characteristics and senescence (Lorin et al., 2006; Scheckhuber and Osiewacz, 2008). Loss of function of the cmETC leads to induction of AOX and decreased ROS. Such strains have dramatically increased longevity but also grow more slowly than wild-type cells—probably due to the presence of only one coupling site for ATP production. Overexpression of AOX results in increased ROS, increased growth rate, and decreased longevity relative to cmETC mutants expressing normal levels of AOX. Thus, the data can be explained by an inverse relationship between ROS levels and longevity, although all factors involved in ROS generation and management are probably not yet understood.

SENESCENCE

Filamentous fungi are generally considered immortal. *P. anserina* is an exception to this rule, as it has a defined vegetative-growth life span (Osiewacz and Scheckhuber, 2002). The senescent syndrome involves a series of events that ultimately affect mitochondrial genome stability and function. Among natural isolates of *Neurospora* species, senescence is primarily detected in strains having mt plasmids that have a propensity to integrate into the mtDNA and is rarely observed in laboratory strains. Yet, like *Podospora*, interactions between nuclear and mitochondrial genetic systems play an important role in senescence, and plasmid-free nuclear senescent mutants that affect mtDNA stability have also been identified. Regardless of its initial cause, senescence in filamentous fungi invariably involves mitochondrial dysfunction and, in most cases, is associated with rearrangements or deletions of mtDNA. The term senescence was initially used to describe the growth of *P. anserina* (Rizet, 1953) and has since been applied to any filamentous fungal culture that shows an initial period of constant vegetative growth before growth slows and stops altogether. Senescence has been documented in several filamentous fungi, but most investigations have been conducted with *Podospora* or *Neurospora* (reviewed by Bertrand, 2000, and Griffiths, 1992).

Non-Plasmid-Related Senescence

While the life span of *P. anserina* strains is a cytoplasmically controlled trait, specific nuclear genes play a role in controlling the onset of senescence (Marcou, 1961; Tudzynski and Esser, 1979). In addition to the GRISEA transcription factor, which influences the rate of senescence in *P. anserina* (described above), other nuclear genes are known to modulate life span (Rossignol and Silar, 1996). For example, deletion of the nuclear gene encoding a dynamin-related protein involved in mitochondrial fission results in a markedly increased life span in *Podospora* that correlates with a severe reduction of senDNA amplification as well as an increased resistance to the apoptosis inducer etoposide (Scheckhuber et al., 2007). In *Neurospora*, two nuclear gene mutants, *natural death* or *nd* (Sheng, 1950) and *senescent* or *sen* (Navaraj et al., 2000), have been shown to cause senescence. The mtDNA of presenescent cultures of both *nd* and *sen* have large deletions and/or gross rearrangements caused by intramolecular recombination events, suggesting that the products of the nuclear nd^+ and sen^+ genes are involved in mtDNA recombination or repair, although it has also been suggested that the product of the *sen* gene could play a role in protein import (Maheshwari and Navaraj, 2008).

Plasmid-Induced Senescence

Studies of *Neurospora* strains that contain the Kalilo linear mtDNA plasmid revealed that repeated vegetative transfer leads to growth defects and senescence (reviewed by Griffiths, 1992). Senescence has also been associated with the linear DNA plasmid Maranhar of *N. crassa* (Court et al., 1991) and variant forms of the Mauriceville and Varkud family of circular retroplasmids (Akins et al., 1986). Curiously, plasmid-associated senescence has not been detected for other plasmid families, such as circular plasmids with a DNA-based replication mode, linear retroplasmids, or RNA plasmids, nor has it been reported in organisms other than *Neurospora* species. While there are plasmid- and strain-specific differences, plasmid-associated senescence generally involves the integration of autonomously replicating forms of the plasmid into the mtDNA. A single integration event may disrupt an essential gene, or multiple integration events, followed by homologous recombination, can lead to large deletions of the mitochondrial genome. Once formed, the defective mtDNAs appear to overtake cultures and exhibit "suppressivity" toward wild-type mtDNAs, to the point that intact (functional) mtDNAs become scarce. This is similar to the behavior of several mitochondrial mutants, such as the "stopper" mutants of *N. crassa* (reviewed by Bertrand, 2000).

One possible explanation for the suppressive effect is that the mtDNAs with large deletions may have a replicative advantage over wild-type DNAs. However, this seems unlikely, as some integrations probably increase the size of the mtDNA. Furthermore, there is one report of senescence in a strain containing a variant form of the Mauriceville

plasmid that was found to be associated with impairment of mitochondrial protein synthesis due to plasmid overreplication but showed no evidence for plasmid integration (Stevenson et al., 2000). This demonstrates that altered mtDNAs are not a prerequisite for suppressivity. The phenomenon of suppressivity as it relates to takeover of cultures by mitochondrial mutations in *N. crassa* has been reviewed previously (Kennell et al., 2004)

In *P. anserina*, an mt plasmid appears to have different effects depending on nutritional status. The linear DNA plasmid pAL2-1 originally was reported to extend longevity in strains in which it had integrated into the mtDNA (Hermanns et al., 1994; Hermanns and Osiewacz, 1996), though later studies found the plasmid to have a neutral effect (van der Gaag et al., 1998). More recently, the pAL2-1 plasmid was shown to decrease life span when cultures were grown on media containing low levels of glucose (i.e., calorie restriction [Maas et al., 2004]). One way to reconcile these contradictory reports is that the integration of the pAL2-1 into the mtDNA interferes with programmed senescence and thus would appear to increase longevity, while as an autonomously replicating element the plasmid has little effect unless strains are subjected to calorie restriction, which extends life span and unmasks the senescence-inducing effect. Interestingly, a recent report found evidence of a cryptic retroplasmid in *N. intermedia* strains that appears to attenuate or suppress senescence in Kalilo strains (Maas et al., 2007). In summary, there appears to be a large range of molecular events that can lead to senescence, but all involve eventual effects on mtDNA and/or mitochondrial function.

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Vacuoles in Filamentous Fungi

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Major contributions of filamentous fungi to the ecosystem include degradation of detritus by saprotrophic fungi, support of plant growth by mycorrhizal fungi, and invasion of humans and major crop plants by pathogenic fungi. Although the fungal vacuole plays a critical role in all of these functions, we are only just beginning to understand what specific genes and proteins are involved, how they are regulated, and the molecular mechanism of their action in formation of this organelle.

Investigators are in good agreement that the vacuole is a dynamic organelle, enclosed by a single membrane, with a variety of structural forms: small vesicles, tubules, and large vesicles. It is an acidic compartment as a consequence of the activity of the vacuolar H^+-translocating ATPase (V-ATPase) in the membrane. It contains high concentrations of basic amino acids, polyphosphate, hydrolytic enzymes, and divalent cations. It has a presumed role in nitrogen and phosphate storage, solute transport, regulation of cellular amino acid metabolism, recycling of macromolecules, osmoregulation, and cytosolic ion and pH homeostasis. The vacuole is an important organelle.

STRUCTURE OF THE VACUOLE

The Vacuolar System as a Motile Tubular Complex

The old textbook view of vacuoles as large round organelles changed to a dynamic picture of connected tubules, vesicles, and some large vesicles as the result of work by Anne Ashford and her colleagues (Shepherd et al., 1993; Cole et al., 1998). Using the ectomycorrhizal fungus *Pisolithus tinctorius*, they observed four zones of vacuolar morphology from the growing hyphal tip: (i) the apical zone, which has few or no vacuoles; (ii) the subapical zone with small ovoid-spherical vacuoles; (iii) the nuclear zone, where tubular vacuoles predominate; and (iv) the basal zone, where large spherical vacuoles are most common. (Fig. 1 through 3 in Hyde et al., 2002, show fine examples of these zones.) The parts appear to form a continuous vacuolar system with numerous small tubes connecting small vesicles, large vesicles, and highly tubulated regions. Most striking is the active nature of the vacuolar system in living hyphae. Tubules extend, retract, fuse with each other and with spherical vacuoles, and can deform (reversibly) into small vesicles. Vesicles may sprout small tubules and appear to slide along larger tubules.

Such a dynamic pleiomorphic tubular vacuolar structure has been documented in many other filamentous fungi, including *Paxillus involutus* (Tuszynska et al., 2006), *Phanerochaete velutina* (Darrah et al., 2006), *Phialocephala fortinii* (Saito et al., 2006), *Gigaspora margarita* (Uetake et al., 2002), *Aspergillus oryzae* (Shoji et al., 2006a), *Neurospora crassa* (Hickey et al., 2004), and several members of the oomycete genus *Saprolegnia* (Allaway et al., 1997).

Visualizing the Vacuolar Complex

The vacuolar structure described above is the product of live-cell imaging in microscopes utilizing fluorescent dyes that accumulate in vacuoles and green fluorescent protein (GFP)- or red fluorescent protein (RFP)-tagged proteins targeted to vacuoles or vacuolar membranes. Both fluorescent microscopes and confocal laser scanning microscopes are widely used. Delights of the live-imaging technology are movies showing the dynamic vacuolar system in action. Examples are included on a CD-ROM produced for educational purposes, which can be obtained from www.fungalcell.org (Hickey and Read, 2003).

The dye preferred by at least two research groups is Oregon green 488 carboxylic acid diacetate (carboxy-DFFDA) 6 isomer (catalog no. 06151; Invitrogen-Molecular Probes, Eugene, OR), which accumulates specifically in the lumen of both tubular and spherical vacuoles (Cole et al., 1998; Hickey et al., 2004). The dye diffuses into cells, where it is colorless and nonfluorescent until esterases cleave off the acetate groups to yield the fluorescent, amine-reactive carboxy-DFFDA. Cole and colleagues (Cole et al., 1997) proposed that in *P. tinctorius* the dye crosses the vacuolar membrane via a nonspecific anion transporter and becomes trapped there. Supporting this hypothesis, probenecid, an anion transport inhibitor, prevented uptake of the carboxy-DFFDA from the cytosol to the vacuolar system.

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Other fluorescent dyes from Molecular Probes are also suitable for staining fungal vacuoles. Earlier studies used 6-carboxyfluorescein diacetate (6-CFDA); it works well but is being replaced by its more stable derivative, carboxy-DFFDA. Kitamoto and coworkers (Shoji et al., 2006a) have used 7-amino-chloromethyl coumarin (CMAC), which is converted to a cell-impermeant conjugate with glutathione and accumulates in fungal vacuoles via glutathione pumps. Another useful dye is FM4-64 because it labels plasma membranes, endosomal membranes, and vacuolar membranes in a progressive manner. Commonly used as an indicator dye for endocytosis, FM4-64 beautifully illuminated the endosomal/vacuolar systems of *A. oryzae* (Shoji et al., 2006a) and *N. crassa* (Hickey et al., 2004) but labeled only plasma membranes and vacuoles in dead or damaged cells in *P. tinctorius* (Cole et al., 1998).

More recently, investigators have been visualizing the vacuolar system with probes containing marker proteins tagged with GFP and RFP. The availability of complete genome sequences for *A. oryzae* and *N. crassa* greatly facilitated these studies. Genes for proteins targeted to the vacuole have been obtained by PCR and cloned into GFP and RFP vectors for transformation to *A. oryzae* and *N. crassa*.

Kitamoto and his colleagues initiated the studies with GFP and RFP in vacuoles of filamentous fungi. Based on studies with *Saccharomyces cerevisiae*, they constructed a strain of *A. oryzae* expressing the fusion protein of carboxypeptidase Y, a protease in the vacuolar lumen, and enhanced GFP (CPY-EGFP) (Ohneda et al., 2002). Unexpectedly, the fluorescence of CPY-EGFP was weak and unclear in cells grown on acidic medium (pH 5.5, which is optimal for growth) but appeared in the lumen of vacuole-

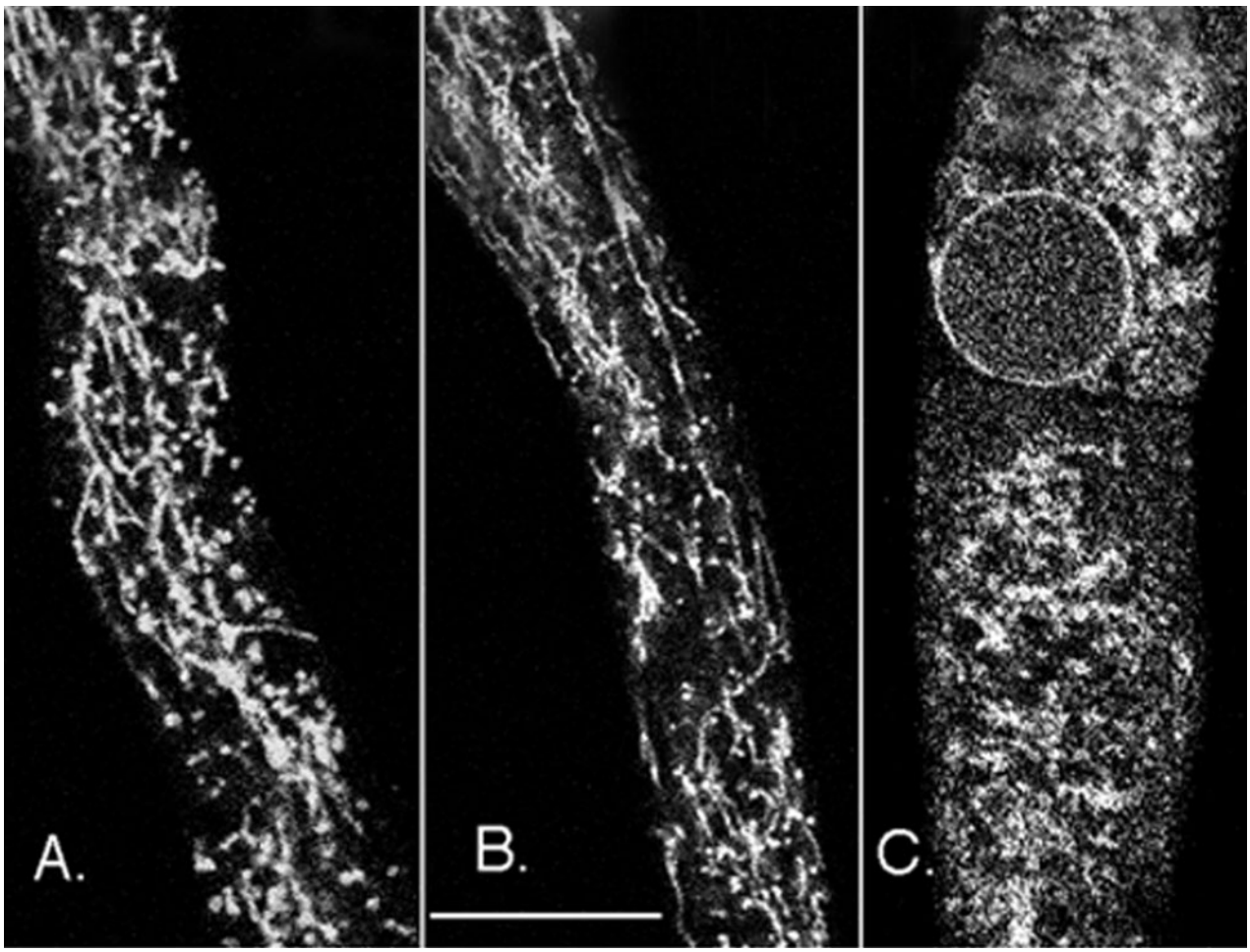

FIGURE 1 Visualizing the vacuole with RFP and GFP. RFP (dsRED) or GFP was fused to proteins predicted to be in the membrane of the vacuole, using the plasmids pMF272 or pMF334 constructed by M. Freitag (Oregon State University, Corvallis). By transformation into *N. crassa,* the recombinant genes were targeted to the *his-3* locus. Images were obtained by confocal microscopy (B. Bowman and E. J. Bowman, unpublished results). (A) RFP dsRED was fused to the N terminus of the CAX protein. (B) RFP dsRED was fused to the N terminus of the VAM-3 protein. In panels A and B the region shown is approximately 100 μm behind the hyphal tip. The vacuolar system consists of tubules and small vesicles. (C) GFP was fused to the C terminus of the A subunit of the V-ATPase (encoded by *vma-1*). The region shown is approximately 2 mm behind the hyphal tip. In this older part of the hypha, the fluorescence is localized to the membrane of a large vacuole and to many small vesicles. In this strain the vacuole appears as a network of tubules and small vesicles nearer the hyphal tip (not shown), as in panels A and B. The bar represents 10 μm, and the scale is the same for all panels.

like structures grown on medium of alkaline pH (8.0). Apparently, EGFP fluorescence is sensitive to low pH. A subsequent paper was more satisfying. Vam3p is a tSNARE protein located on the vacuolar membrane in *S. cerevisiae*. Shoji et al. (2006a) examined vacuolar membranes in strains of *A. oryzae* expressing the fusion protein EGFP-AoVam3p and saw the dynamic, pleiomorphic structure that has become a hallmark of the vacuolar system in filamentous fungi as described above. Thus, AoVam3p became the first protein to be localized on membranes of tubular vacuoles. Beautiful confocal images of the vacuolar membrane system in *N. crassa* are obtained by using fusion proteins between GFP or RFP and VAM-3, proteins involved in calcium transport, such as CAX, and subunits of the V-ATPase (Fig. 1).

A Cautionary Note

Because it is dynamic and flexible, the fungal vacuolar system is subject to variation and change induced by the experimenter. The cells are surprisingly fragile and must be handled with extreme care to avoid damage and artifactual findings. Damage can result from high concentrations of dyes, excessive light intensity, too-long exposure to light, oxygen deprivation, and rough handling of samples. Signs that cells have been perturbed include slowing of hyphal growth, narrowing of the hyphal tip, retraction of the Spitzenkörper from the tip, and changes in organelle morphology (Hickey et al., 2004; Cole et al., 1998). The vacuolar system in particular responds to insult by forming many spherical vesicles with a range of sizes.

Complications in assessing the condition of a sample come from natural variation in the material. Vacuolar form and motility vary with the age of the culture, growth conditions, and the location within the culture (Cole et al., 1998; Ohneda et al., 2002; Shoji et al., 2006a). Furthermore, experimental treatments such as addition of inhibitors may affect growth and morphology of the vacuolar system in the same way as damage does (Hyde et al., 1999, 2002; Tuszynska et al., 2006). When feasible, showing that a response is reversible is desirable.

Another View of the Vacuole

For biochemical studies in vitro, vacuoles can be isolated from filamentous fungi as a pure organellar fraction of round vesicles, approximately 0.2 to 2 μ in diameter. Cramer et al. (1983) developed the original procedure for *N. crassa* in the early 1980s. Cells are grown for 14 to 15 h in aerated liquid culture and lysed in 1 M sorbitol by the action of glass beads in a BeadBeater (BioSpec Products), and vacuoles are then separated from other cell fractions by differential centrifugation. The vacuoles, behaving like osmometers, become very dense and pellet with mitochondria. Centrifugation on a sucrose gradient separates mitochondria from vacuoles, which pellet under a 60% sucrose solution. To isolate vacuolar membranes, the pelleted dense vacuoles are lysed by suspension in a low-osmotic solution (1 mM Tris-HCl, pH 7.5) and then the light vacuolar membranes are pelleted at high speed (Bowman and Bowman, 1997).

After viewing the tubular vacuolar system by microscopy, one might anticipate a large yield of vacuoles and vacuolar membranes from such a preparation. Instead, from a typical cell fractionation procedure we obtain ~200 mg of mitochondrial protein, 20 mg of plasma membrane protein, and 2 mg of vacuolar membrane protein. This is a reminder that the dense tubular vacuolar system occurs only near the growing tips of hyphae, while large spherical vacuoles predominate behind them. The structure of vacuoles in a mass of mycelium grown in the lab, in liquid or on plates, remains to be investigated. Similarly, results from in vitro and in vivo studies should be compared with caution.

MAINTENANCE AND BIOGENESIS OF THE VACUOLAR SYSTEM

Microtubules

For filamentous fungi, the study of formation and biogenesis of the vacuole is a nascent research area. In 1993, Steinberg and Schliwa proposed that all organellar formation and movement in *N. crassa* were microtubule-dependent processes (Steinberg and Schliwa, 1993). Hyde et al. (1999) observed the effects of microtubule inhibitors on the structure of the vacuolar system of *P. tinctorius* visualized with carboxy-DFFDA. The antimicrotubule drug oryzalin largely eliminated the tubular vacuolar system, replacing it with spherical vacuoles. Upon removal of the microtubule inhibitor, the tubular vacuolar system re-formed. Antiactin drugs had little effect on the morphology and motility of the majority of the vacuolar system, but they did cause "massed tubules" at and just behind the tip, possibly because growth was inhibited and tubules piled up as mass flow continued. Electron microscope pictures of freeze-substituted material consistently showed a close alignment of microtubules and vacuoles. Taken together, these results pointed to microtubules, but not microfilaments, as important for determining vacuolar morphology and motility (Hyde et al., 1999).

GTPases

Dynamin-like proteins, which are GTPases, have been implicated in the formation of the interconnected vacuolar system of filamentous fungi. In *P. tinctorius* GTPγS induced a dramatic increase in tubular vacuoles in the first five cells. (Typically, tubular vacuoles are primarily in the first cell in this fungus.) The change was reversible and prevented by preincubation with GDPγS. This behavior was consistent with the action of a dynamin-like protein (Hyde et al., 2002).

A molecular approach was taken in the study of *Aspergillus nidulans*. Kitamoto and his group began choosing homologs of *S. cerevisiae* genes known to be involved in vacuolar biogenesis to evaluate their functions in filamentous fungi. Deletion of the *vps*A gene (*S. cerevisiae* homolog *VPS1*), which encodes a protein related to dynamin, led to severe alterations in the structure of the vacuolar system of *A. nidulans*. Vacuole-like structures, identified by staining with the vacuolar dye 6-CFDA in the disruptant strain, were highly fragmented rather than large and spherical as in the wild-type strain (Tarutani et al., 2001). Deletion of the *ava*A gene (*S. cerevisiae* homolog *VAM4/YPT7*), which encodes a small GTPase in the Rab family, elicited a similar phenotype, i.e., highly fragmented vacuoles (Ohsumi et al., 2002). If Vam4p is fixed in the GDP-bound state by mutation in *S. cerevisiae*, it loses its function in homotypic vacuolar fusion. A strain with the comparable mutation in the *ava*A gene produced highly fragmented vacuoles, consistent with a role for the GDP-bound form of the enzyme in preventing activity. For both of the *A. nidulans* genes the authors suggested that the small vacuole-like compartments might be intermediates in vacuolar biogenesis (Tarutani et al., 2001; Ohsumi et al., 2002). Interestingly, although both *A. nidulans* genes had functions similar to those of their yeast homologs, neither one was able to rescue its respective *S. cerevisiae* deletion strain.

Late Endosomal Markers

Data from other deletion strains led to more complex interpretations (Tatsumi et al., 2007). An *S. cerevisiae* strain lacking Vps24p, a marker protein for late endosomes, grows normally and has intact vacuoles because an alternative route for vacuolar biogenesis is available. In contrast, the homologous deletion in *A. oryzae* resulted in a strain (Aovps24) that grew poorly and contained fragmented and aggregated vacuoles, much like the two *A. nidulans* strains described above. Late endosomal and vacuolar functions appeared to be functionally fused in the *A. oryzae* mutant. Strains with three other late endosomal markers (again by homology to *S. cerevisiae*) fused to EGFP were constructed and examined by confocal microscopy. The EGFP signal appeared as static dot-like structures near vacuoles that were stained by FM4-64 (an endomembrane system dye) but not by CMAC (a vacuolar dye), suggesting an endosomal location only. Expression of the dsRED- and EGFP-tagged late endosomal markers in the Aovps24 strain did not label dispersed dot-like structures but aggregated bodies previously identified as fragmented vacuoles. Thus, the fragmented vacuoles behaved like hybrid organelles with features of both late endosomes and vacuoles. Maybe AoVps 24 is required for the functional separation of these two compartments. The data can be interpreted as evidence for a late endosomal function in vacuolar biogenesis (Tatsumi et al., 2007).

Vacuolar Membrane Marker

AoVam3p was the first protein shown to locate on the membrane of tubular vacuoles in filamentous fungi (Shoji et al., 2006a). The yeast homolog, Vam3p, is a vacuolar membrane syntaxin that regulates vesicular traffic to vacuoles and the homotypic fusion between vacuoles. As anticipated, the fusion protein of EGFP-AoVam3p localized to small punctate structures in the apex, tubular vacuoles behind the apex, and the membrane of large vacuoles further back, like a typical vacuolar network in *A. oryzae*. Surprisingly, it also colocalized with FM4-64. Sequence analysis revealed equal similarity of AoVam3p to two *S. cerevisiae* proteins, Vam3p and Pep12p. (Pep12p is a tSNARE in the late endosome/prevacuolar compartment.) In the absence of a second homolog in *A. oryzae*, it is possible that AoVam3p has the functions and cellular locations of both proteins. Time-lapse imaging of EGFP-AoVam3p for a short period showed multiple changes in vacuolar structure, consistent with a role for the protein in mediating fusion of small vesicular and tubular structures with large spherical vacuoles (Shoji et al., 2006a).

How filamentous fungi form their unique, variable, and vibrant vacuolar system is just beginning to be studied and understood. One lesson from the experiments above is that understanding the biogenesis of the vacuolar system in *S. cerevisiae* cannot be transferred directly to filamentous fungi and that additional studies are essential.

FUNCTIONS OF THE VACUOLAR SYSTEM

Storage of Nitrogen and Phosphorus Compounds

Fungal vacuoles serve as storage reservoirs for high levels of phosphorus and nitrogen in the form of basic amino acids and polyphosphate (polyPi), respectively. R. H. Davis used *N. crassa* in a classic analysis of basic amino acid metabolism and its regulatory role in fungi. The amino acid story and its partner polyamine story are covered in chapter 24 of this volume, and we do not discuss it further here.

The history of polyPi in fungal vacuoles has elements of mystery. What does it do in the vacuole, what form does it take, and how does it get there? Early studies suggested that polyPi served as a counterbalance to the positive charges in the vacuole. Extracts of isolated vacuoles of *N. crassa* were found to contain millimolar concentrations of ornithine (18.1 mM), arginine (13.6 mM), histidine (1.9 mM), and lysine (1.8 mM), as well as spermidine (2.5 mM), Mg^{2+} (2.7 mM), Na^{+} (1.5 mM), and K^{+} (0.7 mM). The polyphosphate-P level (36.7 mM) was sufficient to counterbalance about one-half of the total positive charges (Cramer and Davis, 1984). As a counterion for neutralization of basic amino acids (and other positive ions), the polyPi allows cells to store both P and N compounds in a small volume.

PolyPi was once thought to occur in the form of granules in living cells, but the picture changed with improvements in sample preparation for electron microscopy. Electron microscopic analysis of growing cells of *P. tinctorius*, prepared by anhydrous freeze substitution, showed dispersed phosphorus, largely balanced by potassium ions in large spherical and tubular vacuoles (Cole et al., 1998). X-ray microanalysis of the same samples showed colocalization of phosphorus with potassium. Similar results were obtained with *P. tinctorius* associated with *Eucalyptus pilularis* as an ectomycorrhiza: the polyPi was concentrated in vacuoles of the fungus in dispersed form, not in granules (Ashford et al., 1999).

More recently Saito et al. (2006) used an innovative enzymatic method to localize polyPi in the dark root fungal endophyte, *P. fortinii*. The probe consisted of the recombinant polyPi binding domain of *Escherichia coli* exopolyphosphatase (PPBD) linked to Xpress tag, which could be visualized in confocal microscope images as a fluorescent signal by using anti-mouse Xpress conjugated with Alexa 488 or in transmission electron micrographs as gold particles conjugated with goat anti-mouse-immunoglobulin G antibody interacting with the PPBD mouse-anti-Xpress antibody complex. Again, the polyPi occurred in dispersed form in the fungal vacuoles, with tubular vacuoles appearing to contain less polyPi than spherical or elongated vacuoles.

A new insight into the importance of polyPi storage comes from a study of the corn smut fungus, *Ustilago maydis* (Boyce et al., 2006). Infection of corn by the fungus depends on a morphological transition from budding to filamentous growth. Mutants that limited polyPi accumulation in the vacuole favored the transition to the filamentous form, yet the resultant hyphae had reduced virulence in maize seedlings. One interpretation of these results is that vacuolar polyPi levels play an important part in mediating both morphogenesis and infectivity in this pathogenic fungus.

Vacuolar Calcium

Fungal vacuoles also act as reservoirs for divalent cations, including Ca^{2+}, Zn^{2+}, and Fe^{2+}, to prevent them from reaching toxic levels in the cytosol. In *S. cerevisiae* over 95% of the cellular Ca^{2+} has been reported to be in the vacuole (Eilam et al., 1985). Purified vacuoles of *N. crassa* were found to contain only 0.3 mM Ca^{2+} ions (Cramer and Davis, 1984), and calcium did not colocalize with phosphorus in X-ray microanalysis of *P. tinctorius* (Cole et al., 1998). Electrophysiological experiments demonstrated that the vacuole of *N. crassa* accumulates high concentrations of Ca^{2+} (300 μM) and that this reservoir is largely responsible for maintenance of low concentrations of Ca^{2+} in the cytosol (0.1 μM) (Miller et al., 1990). No calcium was detected in vacuoles of a mutant strain with an inactivated *cax* gene that encodes a putative Ca^{2+}/proton exchanger in the vacuolar membrane (Zelter et al., 2004). Yet the *cax* mutant is not sensitive to high concentrations of Ca^{2+} in the medium. A second *N. crassa* mutant, *nca-2*, is defective

in the homolog of a CaATPase (Pmcp) in the vacuolar membrane of yeast. The *nca-2* mutant retains normal, possibly elevated, levels of vacuolar Ca^{2+} but is sensitive to high amounts (200 mM) of Ca^{2+} in the medium (Abreu, 2003; B. J. Bowman, unpublished results). The phenotypes of the *cax* and *nca-2* mutants suggest that additional components are involved in mediating proper cytosolic Ca^{2+} levels. Because of the universal importance of Ca^{2+} as a signaling molecule, it is important to pursue the question of how the vacuole regulates cellular distribution of Ca^{2+} in a filamentous fungus.

Transport of Solutes

Filamentous fungi that are associated with plants as mycorrhizae or as endophytes are believed to supply nutrients to the plant. That transport of nutrients such as polyPi along the hypha to the plant occurs via the dynamic vacuolar system is a logical and attractive hypothesis. Retrograde transport of carbon compounds from the plant to the fungus could also utilize the vacuolar system. The nature of filamentous growth itself requires the constant translocation of new material to the growing tip. Cytosolic streaming is vigorous and must contribute to growth. Because of its orientation and constant flux, the vacuolar system is a likely conduit as well.

From the Kitamoto lab comes other evidence suggesting that the structure of the vacuolar system points to a role in transport (Shoji et al., 2006a, 2006b). Using the strain of *A. oryzae* that expressed EGFP-AoVam3p to label the vacuolar system (see above), the researchers observed an increase in the amount of tubular vacuolar structure in hyphae that extended onto a glass surface compared to the amount seen in hyphae growing on medium. Perhaps the increased tubular vacuoles served to transport nutrients to the hyphae not in contact with the medium. In mature hyphae, the EGFP fluorescence filled the lumen of the large vacuoles, as though the membrane protein had been internalized. An attractive hypothesis was that the vacuoles degrade cytosolic material by microautophagy (see below) in old vacuoles and recycle it via transport through the tubular connections in the vacuolar system to growing regions of the mycelium.

A detailed study by Darrah et al. (2006) generated the first direct evidence that the tubular vacuolar system functions in transport by following the movement of carboxy-DFFDA through the vacuolar system of *P. velutina*. The researchers monitored the dye's progress by using the technique of fluorescence recovery after photobleaching. They concluded that longitudinal transport through the dynamic, interconnected vacuolar system did occur, over distances of millimeters or even centimeters, and that solute movement could be described by a diffusion model. Moreover, these workers noted that diffusion through the vacuolar system could permit movement of solutes in both directions, thus counteracting the force of mass flow in the cytosol.

Degradative Enzymes

In the wild, filamentous fungi are important agents of recycling, and degrading litter is a major activity. Fungal vacuoles are well known for their high contents of hydrolytic enzymes (Klionsky et al., 1990). Although many of these enzymes have been extensively studied in *S. cerevisiae*, they have to date received much less attention in filamentous fungi. During our efforts to identify subunits of the V-ATPase in *N. crassa*, we isolated an abundant vacuolar protein that proved to be the homolog of proteinase A, the product of the *PEP4* gene in *S. cerevisiae*. Because proteinase A is at the top of an activation cascade, yeast cells containing Pep4p null mutations are deficient in at least three proteases. Inactivation of the *pep-4* gene in *N. crassa* resulted in phenotypes different from those of the mutants in yeast. The *N. crassa* mutant strains were not sporulation deficient, and they were not deficient in protease B and carboxypeptidase Y. Instead, the activities of the other proteases in the *pep-4* mutants were higher than in the wild type, as if the loss of proteinase A was compensated by higher levels of other proteases (Vázquez-Laslop et al., 1996). However, in *Aspergillus niger*, inactivation of the *pepE* gene, which encodes the homolog of *S. cerevisiae* proteinase A, gave a different result. The mutant strain was not deficient in sporulation, but it did exhibit reduced levels of activity for three proteases, similar to the *S. cerevisiae* proteinase A mutant strain (van den Hombergh et al., 1997).

While investigating the incompatibility reaction (described below) in *Podospora anserina*, Paoletti et al. (2001) characterized the homolog of proteinase B, a vacuolar protein involved in autophagy in *S. cerevisiae*. The proteinase B homolog was also purified in *A. niger* (Frederick et al., 1993).

Autophagy

One of the most fascinating and far-reaching roles of the vacuolar system in filamentous fungi is the involvement in autophagy. The process of autophagy has an essential role during starvation, cell differentiation, cell death, and aging in all eukaryotic organisms (Reggiori and Klionsky, 2002; Levine and Klionsky, 2004; Klionsky et al., 2007). One of two cellular pathways for the turnover of proteins and organelles, autophagy takes place in lysosomes of mammals and vacuoles of fungi, both of which have a range of hydrolases capable of degrading all cellular constituents. (The other pathway, proteasome-mediated degradation, is not discussed here.) Microautophagy involves uptake of cytosolic material directly at the vacuolar membrane and is poorly understood. In macroautophagy, bulk cytoplasm or cytosolic organelles are first sequestered in double-membrane vesicles called autophagosomes. The autophagosomes dock with vacuoles and fuse with them. In both sorts of autophagy the membrane vesicles and their contents are released into the vacuole and broken down by vacuolar hydrolases.

Thoroughly studied in *S. cerevisiae*, autophagy has received less attention in the filamentous fungi. However, the basic phenomenon has been demonstrated by using orthologs of marker proteins for autophagy in *S. cerevisiae*. In *S. cerevisiae*, Atg1p is required for the formation of preautophagosomes, and Atg8p moves from the cytoplasm to autophagosomes and to autophagic bodies in the vacuole upon induction of autophagy (Reggiori and Klionsky, 2002). When autophagy was induced by nitrogen starvation in *P. anserina*, the fluorescent GFP-PaAtg8 fusion protein localized to perivacuolar bodies and to the vacuolar lumen (Pinan-Lucarré et al., 2003, 2005). Starvation also induced transcription of two genes involved in autophagy (Pinan-Lucarré et al., 2003). Similar results were obtained with *A. oryzae*. Fusion proteins of EGFP-AoAtg8 and DsRed2-AoAtg8 localized in dot structures similar to preautophagosomes under normal growth conditions and transferred into vacuoles under starvation conditions or following addition of the inhibitor rapamycin, which is convincing evidence that autophagy was induced (Kikuma et al., 2006).

As proposed for other organisms (Levine and Klionsky, 2004), autophagy plays an important role in the differentiation and development of filamentous fungi. Strains of *A. oryzae* with AoAtg8 deleted, which did not accumulate

DsRed-2 from the cytosol in their vacuoles (an indication that autophagy did not occur), were deficient in the differentiation of aerial hyphae and in conidial germination. Furthermore, EGFP-AoAtg8 localized to vacuoles in swollen conidia, germlings, and germ tubes even under nutrient-rich conditions. These (and other) results point to a role for autophagy in differentiation and development (Kikuma et al., 2006). Autophagy also affected differentiation in *P. anserina*. Inactivation of two genes involved in autophagy led to deficiencies in pigmentation, differentiation of aerial hyphae, and development of perithecia (Pinan-Lucarré et al., 2003).

The phenomenon of nonself recognition in filamentous fungi is often associated with autophagy. Also referred to as cell death by incompatibility, vegetative incompatibility, heterokaryon incompatibility, or programmed cell death, it is the subject of chapter 20 in this volume. Cells of growing filamentous fungi fuse with each other to form a mycelial network. However, when two cells that differ genetically at specific loci called *het* or *vic* loci fuse, they undergo cell death by incompatibility. We mention it here because the vacuolar system is intimately involved. As cells of *P. anserina* or *N. crassa* begin to die, the vacuolar system undergoes a morphological change from the tubular network to round vesicles (Pinan-Lucarré et al., 2005; Glass and Kaneko, 2003). The vacuoles fuse into larger vacuoles and then collapse or burst. Because vacuolization is a common feature of cell death by incompatibility, it is likely that vacuolar membrane permeability or rupture releases lytic enzymes and acidifies the cytoplasm, causing cell death in filamentous fungi (Pinan-Lucarré et al., 2007). Destruction of the heterokaryotic cell can be complete within 30 min after hyphal fusion (Glass and Koneko, 2003).

The cause-and-effect relationship between autophagy and cell death by incompatibility is not yet clear. A 2003 study (Pinan-Lucarré et al., 2003) involving *P. anserina* reported that autophagy was induced during cell death by incompatibility. Two genes involved in the autophagic process in *S. cerevisiae* were upregulated during incompatibility, and a GFP-PaATG8 fusion protein accumulated in vacuoles in response to either nitrogen starvation or induction of the incompatible reaction in a self-incompatible strain. A later paper (Pinan-Lucarré et al., 2005) showed that cell death by incompatibility could be experimentally separated from autophagy in *P. anserina*. Deletion of either the PaATG1 gene, involved in early autophagy, or the PaATG8 gene blocked the formation of autophagosomes but failed to prevent cell death or the accompanying vacuolization that is characteristic of both autophagy and cell death by incompatibility. In fact, cell death by incompatibility was accelerated in the deletion mutants, as if autophagy played a protective role.

As suggested by Pinan-Lucarré et al. (2007), the vacuole probably functions both in cell death by incompatibility and in autophagy. The inside-out destruction of the vacuole could be necessary for the induction of cell death by incompatibility, and intact vacuoles could be needed for autophagy to function in turning over cytoplasmic constituents and shielding neighboring cells from destruction.

THE FUNGAL VACUOLE AS A MODEL ORGANELLE FOR STUDIES OF THE V-ATPase

V-ATPases

Discovered in the early 1980s, V-ATPases are large multisubunit enzymes found in all eukaryotic cells. They are present on many components of the endomembrane system and on plasma membranes of specialized cells (Forgac, 2007; Kane, 2006). They have cellular roles in multiple physiological processes, and a number of diseases are associated with their malfunction. V-ATPases were first identified and characterized on vacuolar membranes of fungi and plants. Due to the availability of extensive molecular biology tools and a large number of investigators, *S. cerevisiae* became the premier model organism for investigating the V-ATPase. Later, animal systems became preeminent because of the involvement of V-ATPases in diseases. The filamentous fungus *N. crassa* also served as one of the major model systems for the identification and analysis of the enzyme.

Vacuolar Function and the V-ATPase

The fungal vacuole is an acidic compartment, providing an environment favorable for maturation and activity of hydrolytic enzymes. An electrochemical gradient of protons drives the transport of molecules across the membrane. The V-ATPase in the vacuolar membrane is the driving force for both of these functions. Large enough to be readily seen in an electron micrograph, V-ATPase molecules thickly decorate the vacuolar membrane of *N. crassa* (Fig. 2). *N. crassa* is a favorable model system for study of the V-ATPase because of the ease and economy of growing large numbers of cells, the availability of a procedure for isolating clean vacuolar membranes, and the density of the enzyme on vacuolar membranes.

Structure and Mechanism of the V-ATPase

V-ATPases are composed of two domains that form a ball-and-stalk structure, similar to, but larger than, the related F_OF_1 ATP synthase in membranes of mitochondria, chloroplasts, and bacteria (Forgac, 2007; Kane, 2006; Venzke et al., 2005). The proton-translocating domain of the enzyme, named V_0, contains six types of subunits embedded in the membrane, with a reported stoichiometry of $a_1,c_{4-5},c'_1,c''_1,d_1,e_1$. ATP is hydrolyzed within the V_1 domain of the enzyme, a roughly globular structure connected to V_0 by at least two stalks. V_1 contains eight subunits with a stoichiometry of $A_3B_3CDE_2FG_2H_{1-2}$. Like the F-type ATPase, the V-ATPase functions as a rotary motor. In current models, hydrolysis of ATP by the "motor" (primarily the A and B subunits) drives the rotation in the membrane of the "rotor" (subunits D,F,c,c′,c″, and d). The remaining subunits connect and stabilize the complex structure (Fig. 3). One difference between the subunit content of V-ATPases of fungi and higher eukaryotes has been found. Subunit c′ is present in all fungi but not in other organisms (Chavez et al., 2006; Forgac, 2007).

Genes Encoding V-ATPase Subunits

The first published sequences for subunits of the V-ATPase were for *Daucus carota* and *N. crassa* (Bowman et al., 1988a, 1988c; Zimniak et al., 1988). Today it is known that 14 genes encode the 14 subunits of the V-ATPase in *N. crassa*, making it likely that all the V-ATPases in the cell have the same structure. By contrast, higher eukaryotes express multiple isoforms of some subunits: four for a, two for B, E, H, and d, and three for C and G, making the total number of possible isoform combinations in the enzyme enormous (Forgac, 2007). Most of the isoforms in mammals are expressed in different tissues or in some cases on different membranes within a cell. Even in *S. cerevisiae*, two genes encode one of the subunits, the 100-kDa subunit a of the V_0 sector of the enzyme. The two isoforms localize

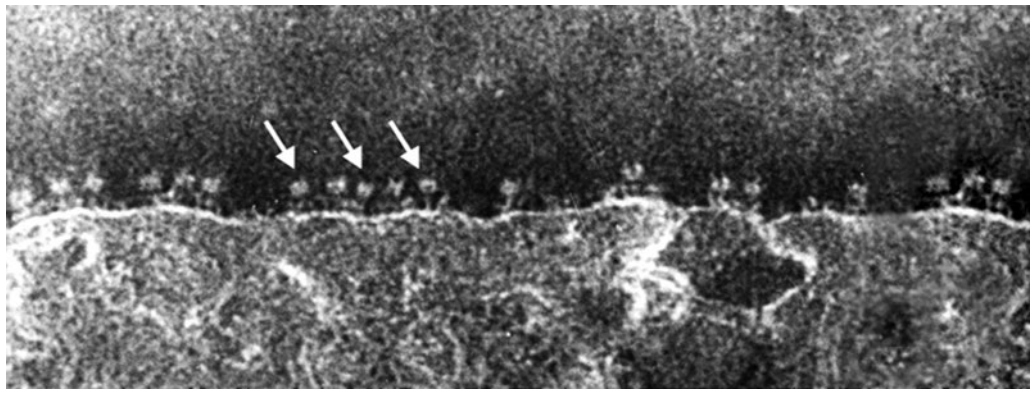

FIGURE 2 Electron micrograph of the V-ATPase in vacuolar membranes. Vacuolar membranes were isolated from *N. crassa*, negatively stained, and examined by transmission electron microscopy as described previously (Dschida and Bowman, 1992). A few of the V-ATPases are indicated by arrows. The globular head is 12 nm wide and is attached to the membrane by a 3-nm-wide stalk.

either to the vacuolar membrane or to the Golgi membrane, and both forms can partially substitute for each other (Manolson et al., 1994).

We have named the genes in *N. crassa* as follows. *vma-1*, *vma-2*, *vma-5*, *vma-8*, *vma-4*, *vma-7*, *vma-10*, and *vma-13* encode the V_1 subunits A, B, C, D, E, F, G, and H, respectively. *Vph-1*, *vma-3*, *vma-11*, *vma-16*, *vma-6*, and *vma-9* encode V_0 subunits a, c, c′, c″, d, and e. The *vma/vph-1* genes have relatively short, multiple introns (two to six introns), which tend to cluster near the ends of the coding region and may be important for high levels of expression (Bowman and Bowman, 2000; Chavez et al., 2006). The genes in *N. crassa* are scattered throughout the genome, raising the question of whether their transcription is regulated in a coordinated manner. This is an interesting point, because variability in numbers of V-ATPase subunits can have severe effects on vacuolar acidification in yeast (Rizzo et al., 2007). Expression levels of all the genes have not been determined, but at least three *vma* genes seem to be transcribed at levels that reflect the relative amount of gene product found in the cell (Wechser and Bowman, 1995). Furthermore, the upstream sequences of four *vma* genes share features with constitutively expressed housekeeping genes (Hunt and Bowman, 1997; Wechser and Bowman, 1995).

Bafilomycin and Concanamycin, Specific Inhibitors of V-ATPases

The most significant contributions from filamentous fungi to the study of V-ATPases resulted from the fortuitous discovery that bafilomycin specifically inhibits V-ATPases of fungi, plants, and animals (Bowman et al., 1988b). The bafilomycins and the related concanamycins are macrolide antibiotics with 16- or 18-membered lactone rings, isolated from *Streptomyces* species (Bowman et al., 1988b; Dröse et al., 1993). Their contribution to the study of V-ATPases is threefold. (i) Sensitivity to these inhibitors was the initial criterion used to identify V-ATPases in multiple organelles and organisms. Currently they are used to investigate numerous cellular processes. (ii) Bafilomycin and concanamycin spawned a new area of drug discovery,

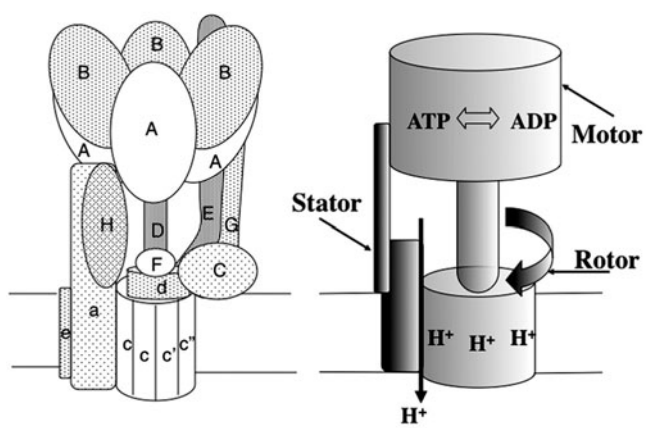

FIGURE 3 Two models of the V-ATPase. As shown on the left, the V-ATPase is composed of 14 different types of subunits, some of which are present in multiple copies. The diagram is modified from the model of the *N. crassa* V-ATPase in Venzke et al. (2005). The model on the right shows the major functional domains. The rotor portion of the enzyme is composed of subunits D, F, d, c, c′, and c″. The A and B subunits form the ATP binding sites and constitute the motor domain. It is not known for certain which subunits form the stator domain.

in which the major targets were V-ATPases involved in osteoporosis and cancer (Bowman and Bowman, 2005; Boyd et al., 2001). (iii) Analysis of mutants resistant to bafilomycin and concanamycin led to a better understanding of the structure and mechanism of the V-ATPase (Bowman et al., 2006).

For reasons that are not clear, mutant strains with an altered V-ATPase that were resistant to bafilomycin or concanamycin in vivo could only be isolated in filamentous fungi. Attempts to isolate resistant mutants in *S. cerevisiae* failed, as did our first attempts with concanamycin as the selective agent in *N. crassa* (Bowman et al., 1997). Selection with bafilomycin was successful. A hallmark of fungal strains with an inactivated V-ATPase gene is the inability to grow at alkaline pH (Nelson and Nelson, 1990). Similarly, wild-type strains cannot grow on medium buffered to pH 7.5 and containing bafilomycin or concanamycin (0.2 to 1.0 μM). We mutagenized *N. crassa* conidia with UV light and selected for strains that were able to grow on medium buffered to pH 7.5 and containing bafilomycin. While a number of conidia germinated, only the strong growers showing wild-type hyphal morphology proved to contain an altered V-ATPase gene (Bowman and Bowman, 2002). In vitro analyses of V-ATPase activity and isolation of the mutated gene confirmed these were V-ATPase mutants: the K_i for inhibition of the enzyme by bafilomycin was increased, and *vma-3*, the gene encoding the proteolipid subunit c in the V_0 domain, was mutated. We named the strains bfr (bafilomycin resistant) strains.

Because bafilomycin and concanamycin have similar structures, we expected the V-ATPases in the bfr strains to be resistant to concanamycin. They were not. However, by subjecting bfr strains to UV mutagenesis and selecting for strains growing at pH 7.5 in the presence of concanamycin, we isolated ccr strains (concanamycin resistant) that were resistant to both inhibitors in vivo and in vitro. The ccr strains all had additional mutations in the *vma-3* gene (Bowman et al., 2004). Altogether we have isolated 55 independent bafilomycin/concanamycin-resistant strains that have amino acid changes at 12 different sites, all in the c subunit of the V-ATPase. Site-directed mutagenesis targeted another 12 residues (Bowman et al., 2006). The increase in resistance to bafilomycin among all the mutants ranged from 2-fold to 450-fold; the increase in resistance to concanamycin ranged from no effect to 97-fold. We hypothesize that both inhibitors bind the same region of subunit c but that concanamycin, which is slightly larger, may have more contact points and bind more tightly.

One goal of the mutagenesis studies was to identify the binding site(s) of bafilomycin and concanamycin on the V-ATPase to determine how the inhibitors act. Subunit c is a highly conserved hydrophobic protein with four predicted membrane helices, an evolutionary duplicate of the two-helix subunit c in F-type ATPases (Mandel et al., 1988). We constructed a model of the arrangement of the V-ATPase helices based on the crystal structure of the c-subunit ring of the F-type ATPase (Stock et al., 1999). Most of the residues implicated in binding bafilomycin/concanamycin clustered together in this model. Positions of the residues supported a model in which the drug-binding site is a pocket formed by helices 1, 2, and 4. We hypothesized that the drugs inhibit by preventing the rotation of the c subunits in the V-ATPase rotor mechanism (Bowman et al., 2004).

We also hoped to generate a good model for the structure of c subunits of the V-ATPase. Our modeling efforts were greatly facilitated by the publication of a crystal structure for the homologous subunits of the V-ATPase in the bacterium *Enterococcus hirae* (Murata et al., 2005). The new model shows the positions of the residues that confer resistance to bafilomycin and concanamycin in a 10-membered subunit c ring. Ten of the 11 mutation sites that confer the highest degree of resistance are closely clustered. They form a putative drug-binding pocket at the interface between helices 1 and 2 on one c subunit and helix 4 of the adjacent c subunit. The excellent fit of the *N. crassa* sequence to the *E. hirae* structure and the degree to which the structural model predicted the clustering of these residues suggest that the folding of the eukaryotic polypeptides is very similar to that of the bacterial protein (Bowman et al., 2006).

Phenotype of V-ATPase Null Mutants

Inactivation of *vma* genes has a myriad of effects on vacuolar function, growth rate, and development. After publishing our conclusion that *vma-1* was an essential gene (Ferea and Bowman, 1996), we stumbled upon a null strain by letting cultures growing from spores in liquid medium sit on the benchtop for 2 weeks. One tube showed weak mycelial growth in the lower half of the medium. (*N. crassa* is a fast-growing fungus. Wild-type strains reach the surface overnight and produce abundant conidia within 4 days.) The rescued mycelium proved to be an authentic *vma* null strain (Bowman et al., 2000). generated by the process of repeat-induced point mutation (see chapter 10 in this volume).

Disruption or replacement of *vma* genes encoding different V-ATPase subunits gives almost identical phenotypes (Bowman et al., 2000; Chavez et al., 2006). Many functions attributed to vacuoles are lost. Vacuoles contribute to pH homeostasis, and *vma* null strains grow poorly in acidic medium (the optimum pH is 5.5 to 5.8), weakly in more acidic medium, and not at all in basic medium (pH 7.2 and above). Vacuoles store large amounts of basic amino acids; *vma* mutant cells have one-fifth the amount of acid-soluble arginine found in wild-type cells. Vacuoles store potentially toxic divalent cations; the presence of 4 mM Zn^{2+} in the medium inhibits growth of *vma* null strains by 85% but does not affect the wild type. As mentioned above, the finding that Ca^{2+} in the medium is less toxic to V-ATPase mutants in *N. crassa* (50% inhibition by 100 mM Ca^{2+} versus 13% inhibition of wild-type growth) than to mutants in *S. cerevisiae* needs to be explained. Vacuoles have a role in osmotic homeostasis, and vacuoles do not pellet as dense organelles in *vma* mutants, apparently having lost the capacity to behave like osmometers. The phenotypes of *vma* null strains provide strong evidence for the involvement of vacuoles in these various processes.

The most severe consequences of inactivating the V-ATPase are on growth and development. The primary effect appears to be on the control of tip elongation and morphology. We measured growth as the increase in colony diameter on an agar surface. The *vma-1* (encodes catalytic subunit A) null strain grew at 0.5 mm/h, eight times slower than the wild type (4.2 mm/h). In the slowly advancing mutant strain, branching is primarily dichotomous and frequent, resulting in a crowded growth front that often looks layered. Aerial hyphae are short and rare. By comparison, the wild type grows mainly by apical extension, producing side branches at less frequent intervals, and forms aerial hyphae that result in a cottony appearance. *vma* null strains cannot differentiate conidia (asexual spores) or form perithecia (female fruiting bodies). However, they can act as a male and contribute a nucleus in a fertile sexual cross.

Fewer than 1% of the spores carrying a *vma* null mutation germinate. We constructed heterokaryons between *vma-1* null mutants of opposite mating types and a helper strain. The heterokaryons grew normally, indicating that the *vma* mutation is recessive. The helper strain contributes to perithecial development but not to ascus and ascospore development. Crossing two *vma* null strains to each other resulted in arrest of ascus development in meiotic prophase (Bowman et al., 2000). The V-ATPase clearly has a prominent and essential role in morphogenesis and development of *N. crassa*. However, little is known about the mechanisms that the vacuole employs to mediate these functions in filamentous fungi.

We have found some interesting differences in the phenotypes of different *vma* mutants. For example, two mutant alleles of *vma-1* (which encodes the A subunit) were generated by repeat-induced point mutation. One of these, named *pvn1*, has a stop codon at amino acid residue 130 (of a total of 607) and is unlikely to make a polypeptide, while the second, *pvn2*, has only four amino acid changes and produces a membrane-bound protein detectable on a Western blot (E. J. Bowman, unpublished results). No V-ATPase activity was detected in membrane fractions from either strain, and both had the phenotype of the *vma* null mutants described above for almost all characteristics (Bowman et al., 2000). However, it was surprising that growth on 1 M sorbitol induced conidiation in the *pvn2* mutant. Although not detectable in vitro, perhaps a very low level of V-ATPase activity was present and accounted for the ability to conidiate. We speculated that sorbitol could desiccate the culture and induce a stress response, which might include upregulating expression of V-ATPase genes. More unexpected was the difference in ascospore viability; *pvn-2* spores germinated as well as the wild type. This could result from a low level of V-ATPase activity, or because the presence of the protein product, even though inactive, is required for spore germination.

vma-11 mutant strains, which are either defective in, or completely lacking, the c′ subunit, have a less severe *vma* null phenotype (Chavez et al., 2006). These strains grow slightly better than the other *vma* null strains. The "extra" growth is inhibited by concanamycin, suggesting that some V-ATPase activity is present. Subunit c′ occurs in V-ATPases of fungi but not other organisms. Perhaps even in fungi it is not completely essential or can be replaced by an extra copy of the closely related c subunit. Like *pvn-2*, the *vma-11* mutants produce conidia when grown on 1.5 M sorbitol. However, *vma-11* strains with nonsense mutations or with complete deletion of the gene exhibit very low levels of ascospore germination (Chavez et al., 2006). A functional V_0 rotor domain (an assembled cluster of c, c′, and c″ subunits) may be critical for ascospore germination. *vma-11* and *vma-16* mutant strains produce few viable ascospores, and despite extensive efforts, a viable spore with an inactivated *vma-3* gene has never been recovered.

Role of the V-ATPase in Vacuolar Morphogenesis

Inactivation of a V-ATPase gene changes the structure of the vacuolar system in *N. crassa*. It was reported that vacuoles in a *VMA* null strain of *S. cerevisiae* are normal (Yamashiro et al., 1990). This was not the case in the *vma-1* null strain of *N. crassa*. Compared with wild-type vacuoles, which are spherical, the vacuoles in the mutant were irregular, often misshapen, and frequently multilamellar (Bowman et al., 2000). This is interesting in view of fascinating recent studies involving *S. cerevisiae*, examining the role of the V-ATPase in regulating vacuolar fusion and fission (Baars et al., 2007). The results from Andreas Mayer's lab show that homotypic fusion of vacuoles requires the physical presence of the V-ATPase (at least the V_0 part) but not H^+-translocating activity. Fission, however, does require V-ATPase pump activity. The investigators propose that vacuolar morphology results from an equilibrium of competing fission and fusion reactions, with the ratio of the rates determining the outcome. Vacuolar morphology was visualized with FM4-64 in deletion strains lacking Vma1p, Vma3p, and Vma6p (Baars et al., 2007). They contained one enlarged vacuole/cell, suggesting that fusion was dominant. By analogy, we suggest that similar processes determine the structure of vacuoles in the *vma-1* null strain of *N. crassa*.

V-ATPase and Autophagy

In *S. cerevisiae*, the V-ATPase plays an essential role in autophagy (Reggiori and Klionsky, 2002). The breakdown of engulfed material by vacuoles requires normal acidification of the vacuole, both to provide the acid pH that is optimal for degradative enzymes and to facilitate the autocatalytic cleavage and activation of proteinases A and B. These proteases initiate a proteolytic cascade that leads to the activation of most vacuolar hydrolases. The V-ATPase achieves the acidification. The contribution of the V-ATPase to autophagy in filamentous fungi has not yet been investigated. Such studies will indicate whether the vacuole plays a conserved function in regulating autophagy in yeasts and filamentous fungal species.

SUMMARY

The former image of the vacuole as the junkyard of the cell in filamentous fungi has been replaced by a picture of a constantly changing, multifunctional meshwork of vesicles and tubules. The vacuolar system is highly variable in appearance at different locations within the mycelium and in response to different growth conditions. We know almost nothing about what controls and regulates the structure of the vacuole. Is it as functionally compartmented as it is structurally diverse? Although we have a good idea of what the vacuolar contents are, we are comparatively ignorant as to how they get there, how they are stored, how they are used, and how they are moved about. The nature of tip growth necessitates the transport of materials over long distances in the mycelium; however, the role of vacuoles in polarized growth has been largely ignored. Our understanding of autophagy in filamentous fungi is primitive, yet we suspect that it plays a critical role in recycling resources to support tip growth.

Several labs are introducing new approaches to study the dynamic behavior of the vacuolar system of filamentous fungi. One example is the work by Darrah et al. (2006), in which fluorescence recovery after photobleaching and mathematical modeling are combined to determine the connectivity of membranous compartments in vivo. Other methods are being developed by Watkinson et al. (2005). Using a combination of photon counting scintillation imaging and stable-isotope nuclear magnetic resonance, this group's goals are to observe how a transport system develops and is altered and to follow nitrogen in a mycelial network growing in a "realistic soil."

Molecular approaches similar to that pioneered by the Kitamoto group to take advantage of genome sequence data from filamentous fungi should facilitate rapid progress in understanding vacuolar structure and function. Homologs of *S. cerevisiae* genes with known functions in vacuolar biogenesis, autophagy, and ion transport are excellent candidates for

identifying the genes involved in these processes in filamentous fungi. Genetic studies of strains with selected genes deleted either singly or in combination with others will allow us to evaluate their roles in the cell. Confocal laser scanning microscopy of fusion proteins with GFP and RFP or other tags will reveal the cellular locations of these proteins. We can anticipate finding novel adaptations in how filamentous fungi use these genes and their products to support their unique lifestyle.

One of the most fascinating questions about the vacuolar system is whether it is structurally and functionally compartmentalized. Are endocytic and lysosomal functions in separate vesicles or membrane segments? Are calcium and arginine enclosed together or in specialized vacuoles? Do forward transport and retrograde transport occur simultaneously in the same tubule? Are the three vacuolar calcium transporters in the same or different regions of the vacuolar complex? Can autophagosomes fuse with any region of the vacuolar system? Methods used by Valkonen et al. (2007) might be applied to such questions. Using fluorescence lifetime imaging microscopy in combination with Foerster resonance energy transfer, these authors were able to demonstrate for the first time the spatial and functional separation of two SNARE proteins on the plasma membrane in a filamentous fungus.

In addition to being intriguing for their contribution to unique growth habits, the vacuoles of filamentous fungi have been valuable as model systems, especially in two areas. First is their role in arginine metabolism: the compartmentalized storage, the regulation of cytosolic concentrations, and the mobilization in times of need (chapter 24). Second is their contribution to the discovery of V-ATPases and to studies of the structure and function of this major class of enzymes. The investigations performed in recent years demonstrate that the vacuole is a dynamic organelle with many important roles in metabolism, growth, and development. Although it is the most visible organelle in fungal hyphae, the vacuole has not yet received the attention it deserves.

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15

Peroxisomes in Filamentous Fungi

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Peroxisomes are single membrane-bound organelles present in most eukaryotic organisms. They are normally round and small ($\leq$1 μm), but they can be considerably larger and can adopt elongated or branched shapes. Peroxisomes typically contain a number of oxidoreductases that produce hydrogen peroxide (H_2O_2) (hence their name) as well as enzymes for reactive oxygen species (ROS) disposal. They are implicated in several metabolic functions that vary not only between organisms but also with cell and environmental pressures. They are highly dynamic and act in close association with other organelles.

Peroxisomes are involved in the regulation of diverse developmental processes, such as female/male gametophyte recognition during the fertilization of flowering plants (Boisson-Dernier et al., 2008) and neuronal differentiation and migration during brain development in mammals (reviewed by Faust et al., 2005). Moreover, as illustrated in plants, peroxisomes provide an important source of diverse signaling molecules (Nyathi and Baker, 2006). In humans, peroxisomes are required for various developmental events and deficiencies result in multiple severe anomalies that are often lethal (Steinberg et al., 2006; Wanders and Waterham, 2006). Despite significant research using various model organisms, peroxisome function is not completely understood, in particular, their impact on development. In this respect, filamentous fungi have been increasingly used as model organisms to study peroxisomes. In this chapter, we present an overview summarizing the current knowledge of the function of peroxisomes in filamentous fungi.

PEROXISOME DYNAMICS IN FUNGI

Peroxisomes are present in most major fungal groups. The main exceptions are the groups of anaerobic fungi that are also devoid of true mitochondria, such as Microsporidia (Cavalier-Smith, 1987). Similarly, there is no conclusive evidence for their existence in the rumen fungi Neocallimasticales, whose microbody-like organelle, the hydrogenosome, is now recognized as derived from mitochondria (Embley et al., 2003). The recognition of these lineages as fungi (James et al., 2006) indicates that their peroxisomes were lost secondarily, which is further supported by the existence of orthologs of some *pex* genes in microsporidian genomes (Fedorov and Hartman, 2004).

Peroxisome Assembly, an Overview

Peroxisome formation among diverse eukaryotic organisms shares a common basic biogenetic process mediated by a number of conserved proteins known as peroxins (for which the Pex acronym has been adopted [Distel et al., 1996]). Proteins controlling peroxisome dynamics are classified according to their involvement in three major processes (Table 1): (i) biogenesis of peroxisome membranes and insertion of peroxisomal membrane proteins (PMPs), (ii) peroxisome matrix protein import, and (iii) peroxisome division and inheritance.

Both peroxisome membrane and matrix proteins are synthesized in the cytosol and are sorted to peroxisomes to be inserted into the membrane or translocated to the peroxisome matrix, respectively. Three proteins are known to control the biogenesis of the peroxisome membrane and the insertion of PMPs (Table 1), and most cells lack detectable peroxisome membranes if any of these peroxins are absent (reviewed by Fujiki et al., 2006). In contrast, absence of the peroxins implicated in the import of the peroxisome matrix proteins results in (empty) peroxisomal remnants. The latter process relies on a larger set of peroxins (Fig. 1). Pex5 and Pex7 are the soluble receptors recognizing peroxisome matrix proteins in the cytosol (by means of their PTS1 or PTS2 targeting sequences, respectively), whereas the docking site at the peroxisome membrane is provided by the peroxins Pex14, Pex13, and, in yeasts, Pex17p (the docking complex). These proteins, together with RING finger domain-containing PMPs Pex2, Pex10, and Pex12, form a larger complex known as the importomer; this complex probably constitutes the translocator for peroxisome matrix proteins. In *Saccharomyces cerevisiae*,

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TABLE 1 Predicted peroxins of *P. anserina*[a]

Peroxin	*P. anserina* coding sequence	Characteristic	Main function(s)	Comments[a]
Biogenesis of peroxisome membranes and peroxisomal membrane protein insertion				
PEX3	Pa_7_8080	PMP	Peroxisome membrane anchor of PEX19, importomer linking in *P. pastoris*	M, F
PEX16	Pa_1_21920	PMP, intraperoxisomal in *Y. lipolytica*	PMP targeting, peroxisome proliferation in *Y. lipolytica*	
PEX19	Pa_2_10100	CAAX box	Receptor/chaperone of PMPs	M, F
Peroxisome matrix protein import				
PEX1	Pa_1_6740	AAA-type ATPase	Peroxisome dislocation of Pex5p	A, ER, F
PEX2	Pa_1_2750	PMP, RING finger domain	Importomer protein	A, ER, F
PEX4	Pa_1_9240	E2 ubiquitin conjugation enzyme	Monoubiquitination of Pex5p	A, ER, F
PEX5	Pa_1_18440	Tetratricopeptide repeats, WxxxF/Y motifs	PTS1 receptor, PTS2 coreceptor (plants and mammals)	A, ER, F
PEX6	Pa_1_9330	AAA-type ATPase	Peroxisome dislocation of Pex5p	ER, F
PEX7	Pa_6_3500	WD-40 repeats	PTS2 receptor	F
PEX8	Pa_7_9670	Leucine zipper motive	Importomer linking protein	
PEX9			ORF incorrectly identified	
PEX10	Pa_1_4170	PMP, RING finger domain, E3 ubiquitin ligase	Importomer protein, polyubiquitination of Pex5p	M, A, ER, F
PEX12	Pa_3_3790	PMP, RING finger domain	Importomer protein	M, F
PEX13	Pa_1_4570	PMP, Src homology 3 domain	Importomer (docking) protein, membrane anchor of PEX14	F
PEX14	Pa_4_8910	PMP, PxxP-motif	Importomer (docking) protein, membrane anchor of receptors	A, F
PEX14/17	Pa_3_5160	Pex14-N-term conserved region	Predicted docking protein in filamentous fungi	
PEX15		PMP	Membrane anchor of PEX6 in *S. cerevisiae*	
PEX17		PMP	Docking protein in *S. cerevisiae*	
PEX18		WxxxF/Y motifs	PTS2 coreceptor in *S. cerevisiae*	
PEX20	Pa_4_7290	WxxxF/Y motifs	PTS2 coreceptor	
PEX21		WxxxF/Y motifs	PTS2 coreceptor in *S. cerevisiae*	
PEX22		PMP	Membrane anchor of PEX4	
PEX22-like	Pa_6_4620	1 predicted TM segment	Predicted peroxin	
PEX26	Pa_7_1760	PMP	Membrane anchor of PEX6 in *Homo sapiens*	
Peroxisome division/proliferation				
PEX11	Pa_4_9300	PMP	Peroxisome proliferation/division	F
PEX11B	Pa_4_7960	3 predicted TM segments	Predicted peroxin	
PEX11C	Pa_7_5380	No predicted TM segments	Predicted peroxin	
PEX23	Pa_4_3500	PMP dysferlin motifs	Peroxisome proliferation; protein import?	F
PEX23-like	Pa_2_660	No predicted TM segments	Predicted peroxin	
PEX24	Pa_2_620	PMP	Peroxisome size and number; protein targeting?	F
PEX25		PMP	Peroxisome size and number	
PEX27		PMP	Peroxisome size and number	
PEX28	Pa_2_620	PMP	Peroxisome size and number; *S. cerevisiae* Pex28p ortholog of *Y. lipolytica* Pex24p	
PEX29		PMP	Peroxisome size and number	
PEX30	Pa_4_3500	PMP, dysferlin motifs	Peroxisome number negative regulator; *S. cerevisiae* Pex30p ortholog of *Y. lipolytica* Pex23p	
PEX31		PMP, dysferlin motifs	Peroxisome size negative regulator	
PEX32		PMP, dysferlin motifs	Peroxisome size negative regulator	

[a]Abbreviations: M, marker peroxin, present in all peroxisome-containing organisms and absent from organisms devoid of peroxisomes (Schluter et al., 2006); A, ancient peroxin, present in the common ancestor of peroxisome-containing organisms (Gabaldon et al., 2006); ER, peroxin presenting similarity to an ER-associated degradation pathway protein; F, peroxins present in major taxa of Fungi (see the text).

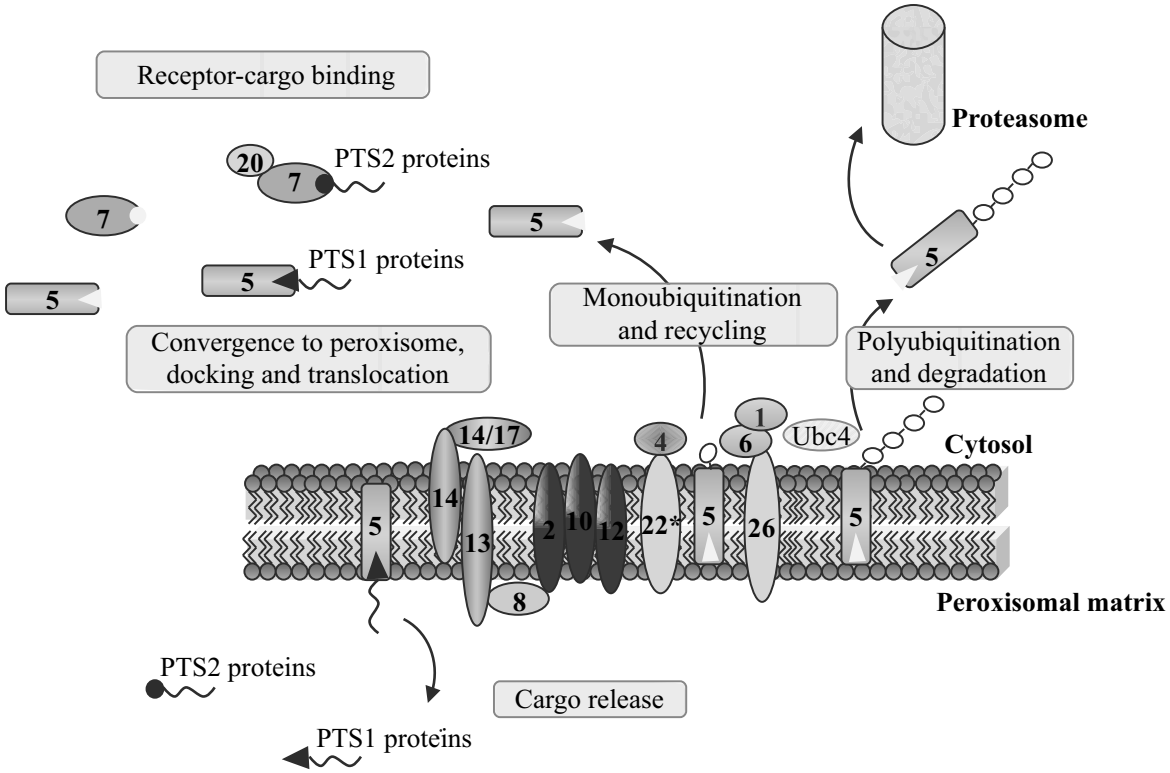

FIGURE 1 Schematic representation of the putative peroxisome matrix protein import pathway in filamentous fungi. The recognition of the peroxisomal matrix proteins by Pex5 and Pex7 receptors occurs in the cytosol (Pex5 and Pex7 recognize PTS1 and PTS2, respectively); the cargo-receptor complexes then dock at the peroxisomal membrane (where both import pathways converge) and are then translocated. These last processes require the importomer, which is composed of two subcomplexes linked by Pex8: the docking complex (formed by Pex14, Pex13, and Pex14/17) and the RING finger complex (formed by Pex2, Pex10, and Pex12). After cargo release in the peroxisomal matrix, Pex5 receptors are either monoubiquitinated (by Pex4) or polyubiquitinated (by Ubc4). The release of the Pex5 ubiquitinated form requires Pex1 and Pex6, AAA-type ATPases anchored in the peroxisomal membrane by Pex26. When polyubiquitinated, Pex5 receptors are then degraded by the proteasome; when monoubiquitinated, they are recycled back to engage in another round of import. The asterisk in "Pex22*" denotes that in filamentous fungus genomes a Pex22-like protein is usually present rather than a true Pex22 ortholog (see also Table 1).

Pex8p is the peroxin bringing the docking and RING finger complexes of the importomer together (Agne et al., 2003). Peroxisome matrix proteins are translocated together with their receptor Pex5 or Pex7; after cargo release in the peroxisome matrix, these receptors are cycled back to the cytosol to be reutilized in additional rounds of import. Export of Pex5 is dependent on a ubiquitination activity that marks the receptor for recycling (after Pex4-mediated monoubiquitination) or degradation (after Ubc5/6-mediated polyubiquitination). The ubiquitinated receptors are released from the peroxisome membrane by an AAA-type ATPase-heteromultimeric complex formed by Pex1 and Pex6 and anchored to the peroxisome membrane by Pex26 (reviewed by Platta and Erdmann, 2007).

The Formation of Fungal Peroxisomes

Peroxin genes have a eukaryotic origin. There is a highly conserved array of peroxins (Table 1), which likely represent the essential core of peroxisome biogenesis factors of early eukaryotes. Notably, a large proportion of the most ancient peroxins exhibits similarity to proteins of the endoplasmic reticulum (ER)-associated degradation pathway, suggesting a common evolutionary origin for peroxisomes and the ER (Gabaldon et al., 2006; Schluter et al., 2006). With the exception of Microsporidia, which lack most *pex* genes (Schluter et al., 2006), fungi from all major taxa for which a genome sequence is available (Chytridiomycota, zygomycetes, Ascomycota, and Basidiomycota) also contain orthologs of Pex6, 7, 11, 13, 23, and 24 (Kiel et al., 2006; our unpublished observations). Consequently, these proteins probably reflect the essential peroxisome biogenesis proteome of the last common ancestor of fungi. As exemplified for *Podospora anserina* (Table 1), peroxin proteomes of present-day fungi are rather more complex. The analysis of the peroxin distribution among phylogenetically diverse fungi (see below) indicates that this was probably also true for early fungi and that many peroxin genes were replaced and/or recruited during the course of fungal evolution (for exhaustive analyses of fungal peroxins see Kiel et al., 2006, and Kiel and van der Klei, 2009).

In fact, some of the key components required for peroxisome assembly have so far been characterized only in fungi. In some cases, such as the importomer bridging protein Pex8, there is no known ortholog beyond fungi (Kiel et al., 2006; Rayapuram and Subramani, 2006). In contrast, some highly conserved peroxins were lost and/or replaced in the course of fungal evolution; this is the case for some peroxins implicated in the export of receptors. Pex4 is absent from some basidiomycetes, and its anchoring protein, Pex22, is absent from most filamentous fungi. The role of Pex22 in some taxa may be fulfilled by a Pex22-like protein. Similarly, the mostly ubiquitous Pex6-anchoring peroxin, Pex26, is absent from some basidiomycetes and saccharomycetes; however, in the latter group, this has been possibly replaced by Pex15. Another Saccharomycotina-exclusive protein is the docking component Pex17p, but a gene putatively encoding the PEX14/17 hybrid peroxin is present in the genome of most filamentous fungi (Kiel et al., 2006; L. Peraza-Reyes, unpublished observations).

The Dynamics of Peroxisomes in Filamentous Fungi

The ER is now known to play an important role in the biogenesis of peroxisomes (reviewed by Titorenko and Mullen, 2006); however, the population of peroxisomes in a cell is largely controlled by the division of preexisting peroxisomes. Peroxisome division proceeds by three mechanistically different events: peroxisome elongation, constriction, and fission (exhaustively reviewed by Fagarasanu et al., 2007). One of the most conserved peroxins implicated in peroxisome division is Pex11. This protein is required for the proliferation and elongation of peroxisomes and is present in different isoforms in filamentous fungi, along with other proteins implicated in peroxisome proliferation such as Pex23 (Table 1) (Kiel et al., 2006). The archetypical Pex11 protein is indeed implicated in peroxisome proliferation (Kiel et al., 2005; Hynes et al., 2008), but whether additional isoforms are involved in peroxisome dynamics remains to be established. Nevertheless, in other eukaryotes it is common to observe different organism-specific Pex11 isoforms controlling the proliferation of peroxisomes in response to different stimuli, in specific cellular types or in distinct peroxisomal subtypes (Schrader and Fahimi, 2006; Orth et al., 2007, and references therein). The presence of these Pex11 and Pex23 protein families in filamentous fungi possibly reflects some peroxisomal specialization toward the filamentous lifestyle.

Organelle distribution and movement in cells normally require coordination between the actin and microtubule elements of the cytoskeleton. Peroxisomes are highly dynamic organelles in fungi. Their movement and segregation in the budding yeast *S. cerevisiae* require an active interplay with the cytoskeleton, whereas in the fission yeast *Schizosaccharomyces pombe*, their distribution seems to be independent of the cytoskeleton and instead depends on their interaction with mitochondria (Jourdain et al., 2008). In *S. cerevisiae*, *Yarrowia lipolytica*, and plants, peroxisomes are associated with the actin cytoskeleton, whereas they interact with microtubules in mammalian cells (Schrader and Fahimi, 2006; Chang et al., 2007, and references therein). We would expect the peroxisome distribution in hyphae to depend largely on their association with the cytoskeleton, due to polarized growth of filamentous fungi, but this area has not been sufficiently studied. Preliminary experiments with *P. anserina* show that peroxisome movement and distribution in asci are not drastically affected by microtubule-disrupting drugs (V. Berteaux-Lecellier and D. Zickler, unpublished observation). However, this is not necessarily true for other fungi. In many aspects, peroxisome division and positioning parallel those of mitochondria. The mitochondria-cytoskeleton association is rather divergent in filamentous fungi; for example, in fungi such as *Aspergillus nidulans*, mitochondrial movement does not require functional microtubules, whereas in others, such as *Neurospora crassa*, this movement is mainly microtubule dependent (Westermann and Prokisch, 2002). It would be interesting to determine if a partitioned cytoskeleton association is also observed for fungal peroxisomes.

A PEROXISOME-RELATED ORGANELLE OF FILAMENTOUS FUNGI: THE WB

Hyphae are the major mode of cellular organization in fungi, and in most species these filamentous cells are compartmentalized by perforate septa that allow intercellular cooperation and communication. Due to the syncytial growth of filamentous fungi, the wounding of hyphae can lead to a severe loss of cytoplasm and subcellular organelles if the plasma membrane or a nearby septum is not rapidly sealed. Woronin bodies (WBs) are highly specialized peroxisome-related organelles that are unique to filamentous fungi and function to seal the septal pore in response to cellular damage (Trinci and Collinge, 1974; Markham and Collinge, 1987) (Fig. 2). In addition, WBs are also required for efficient pathogenesis (see "Peroxisomes in Predatory and Pathogenic Fungi" below for details), survival during nitrogen starvation (Soundararajan et al., 2004), and conidiation (Simon et al., 2005) in various fungi. Since their discovery by Michael S. Woronin (Woronin, 1864), WBs have been observed by electron microscopy in a variety of euascomycetes and now have been identified in more than 142 species of filamentous fungi (Markham and Collinge, 1987; Würtz, 2007). They appear to be hexagonal to spherical electron-opaque structures bound by a single membrane and have been reported to be between 100 and 750 nm.

The WB was first purified from *N. crassa*, and this allowed the identification of a key WB-associated protein, HEX-1 (Jedd and Chua, 2000; Tenney et al., 2000). Induced hyphal wounding in *hex-1* mutants of *N. crassa*, *Aspergillus oryzae*, and *Magnaporthe grisea* leads to substantial macroscopic bleeding (Jedd and Chua, 2000; Tenney et al., 2000; Soundararajan et al., 2004). Analysis of the HEX-1 crystal structure reveals the existence of three intermolecular interfaces promoting the formation of a three-dimensional protein lattice. Consistent with these data, self-assembly is disrupted by mutations in intermolecular contact residues (Yuan et al., 2003). Production of this assembly-defective HEX-1 mutant protein results in aberrant WBs, which possess a soluble noncrystalline core. This mutant allele also fails to complement an *hex-1* deletion in *N. crassa*, demonstrating that the HEX-1 protein lattice is required for WB function.

HEX-1 possesses a functional peroxisome-targeting signal (PTS1), suggesting that WBs are related to peroxisomes (Jedd and Chua, 2000; Managadze et al., 2007). Expression of *N. crassa* GFP-HEX-1 in wild-type mycelia stains WBs and peroxisomes in a targeting signal-dependent manner (Managadze et al., 2007). The *hex-1* deletion mutant grows normally with oleate as the sole carbon source, a clear indication that WBs are not associated with the peroxisomal pathway of fatty acid β-oxidation (see "Ubiquitous Functions" below for details) and thus are not presumably associated with peroxisomal metabolism. In *N. crassa*, nearly all peroxisome assembly mutants are defective in WB biogenesis (Managadze et al., 2007; Liu et al., 2008). Using the *N. crassa*

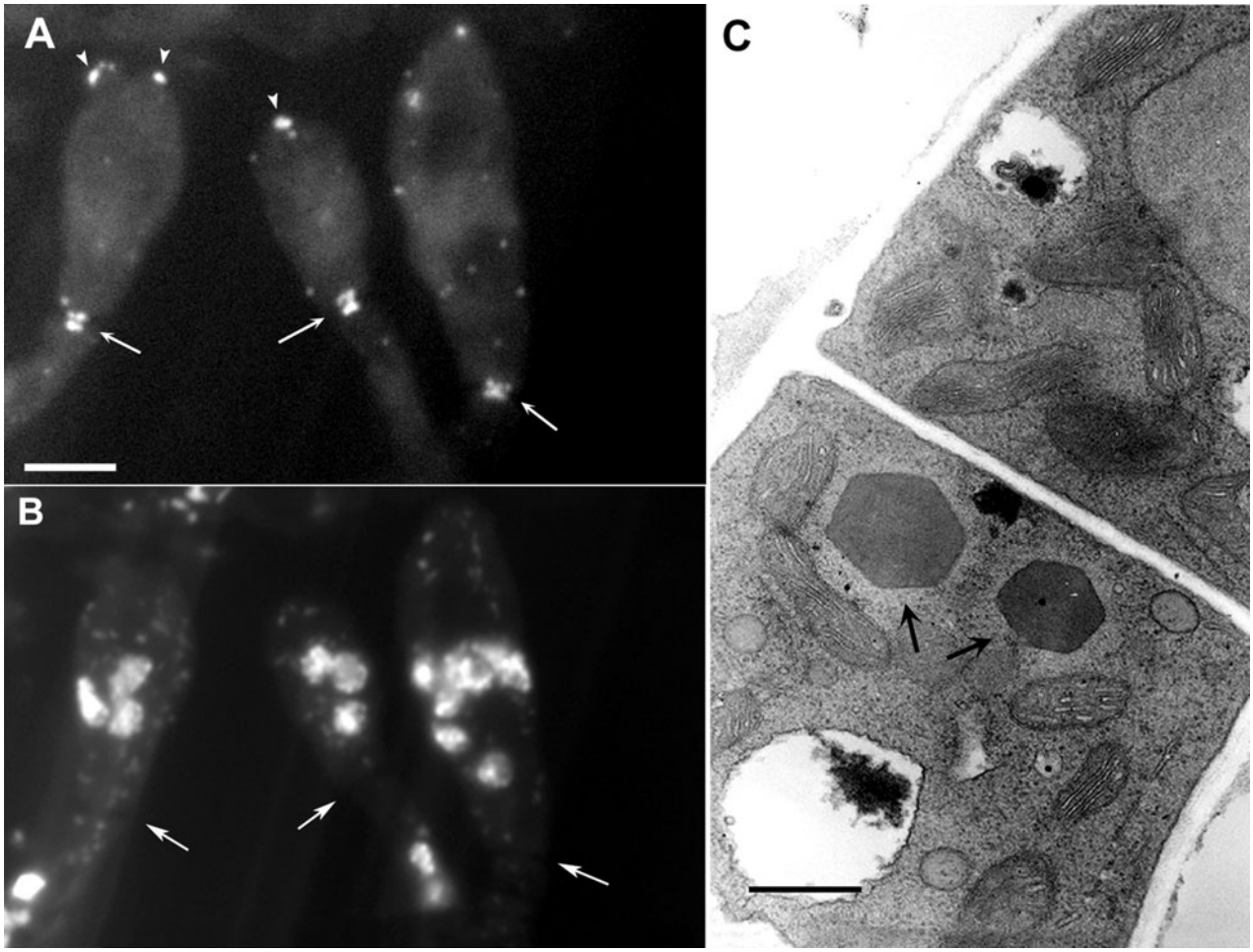

FIGURE 2 Woronin bodies. The WB is a specialized peroxisome of filamentous fungi that function in sealing the septal pores that communicate hyphal compartments. (A) *P. anserina* WBs delimiting apical cells of paraphysae are indicated by arrows; note the recently formed WBs at the tip of the cell (arrowheads). (B) Corresponding DAPI (4′,6-diamidino-2-phenylindole)-stained micrograph. Arrows indicate the positions of the septal pores. (C) In fungi like *N. crassa*, the WB structural lattice is reflected by the hexagonal shape that WBs adopt. In the image, two WBs (arrows) before plugging a septal pore are shown (note that the septal pore is not in the same plane as WBs and is not visible here). *P. anserina* WBs were stained by anti-hex1 antibody, a kind gift from G. Jedd. The transmission electron micrograph in panel C was done by Jorge Sepulveda and L. Peraza-Reyes, courtesy of Wilhelm Hansberg's laboratory (UNAM, Mexico). Scale bars, 5 μm (panels A and B) and 500 nm (panel C).

Δpex14 mutant, impaired in peroxisomal import, Managadze et al. (2007) have shown that HEX-1 remains cytosolic and that WBs are not formed. Furthermore, subcellular fractionation studies demonstrate that PEX14 is not localized to WBs in appreciable amounts, but rather is associated with peroxisomes. Together these observations suggest a transient peroxisomal residence of HEX-1 en route to WBs.

Liu et al. (2008) have recently shown that fungal peroxisomes create WBs through a process that couples the self-assembly of HEX-1 in the peroxisomal matrix with the assembly of the Woronin sorting complex (WSC), a PMP22/MPV17-related peroxisomal membrane protein, at the peroxisomal membrane. HEX-1 crystals are formed but fail to associate with the peroxisomal membrane in *wsc* mutants and instead move randomly in the matrix. In a wild-type strain, as the formation of the HEX-1 crystal proceeds in the peroxisomal matrix, WSC interacts with HEX-1 and keeps the HEX-1 crystal bound to the peroxisomal membrane. The HEX-1 crystal is then tightly surrounded by the peroxisomal membrane as a result of the WSC-HEX-1 interaction. This should ultimately lead to WB budding off. Organelle sizes and their WSC membrane content increase simultaneously with the entrance of HEX-1 into the peroxisomal matrix. Moreover, Liu et al. (2008) have shown that WSC overproduction promotes a cellular cortical association of peroxisomes in the absence of HEX-1. All these observations have led to the suggestion that WSCs have a dual function in WB morphogenesis and inheritance: the sorting of HEX-1 to a defined region within peroxisomes and the cellular cortical association of the resulting WB.

WBs are formed in the apical regions of the growing hyphae of filamentous fungi and are then transported from the apex to the basal regions (Momany et al., 2002). The spatial localization of *hex-1* gene expression has been examined, and WB formation has been directly observed, using time-lapse confocal microscopy, by Tey et al. (2005). The results show that apex-localized gene expression is a key determinant of spatially restricted WB assembly.

VPS1, DNM1 (dynamin-related proteins), and FIS1 (involved in DNM1 function) promote membrane constriction and fission in various intracellular compartments, such as peroxisomes (Hoepfner et al., 2001; Koch et al., 2004; Kuravi et al., 2006) or mitochondria (Mozdy et al., 2000; Hoppins et al., 2007). In *N. crassa*, the deletion of the corresponding genes showed that nascent WBs are less apparent and that the HEX-1 lattice in mature WBs is generally smaller than those observed in the wild-type control (Liu et al., 2008). On the other hand, if HEX-1 is heterologously expressed in the *S. cerevisiae vps1 dnm1* double-deletion strain, formation of WBs is almost completely blocked (Wurtz et al., 2008). Thus, WB formation in the *N. crassa* double-mutant strain may be affected more than that in single mutants, and at least, DNM1 and VPS1 may be required for the differentiation of WBs from peroxisomes (Liu et al., 2008).

Sordaria macrospora PRO40 (Masloff et al., 1999) (essential for the formation of fruiting bodies and thus for the sexual life cycle) and its *N. crassa* ortholog, SO (Fleissner et al., 2005) (necessary for hyphal fusion and female fertility), define a class of proteins present only in filamentous ascomycete fungi that appear to be involved in the response to hyphal injury. Moreover, partial colocalization of PRO40 with HEX-1 has been described in *S. macrospora* (Engh et al., 2007). However, the integrity of WBs is not affected in *pro40* mutant strains. In *N. crassa*, the SO protein localizes to septal plugs and contributes to, but is not essential for, septal plugging (Fleissner and Glass, 2007). Further investigations are required to determine whether the endogenously expressed SO/PRO40 protein is present in WBs. If this turns out to be the case, a novel function for WBs in fruiting body formation may be envisaged.

PEROXISOME METABOLISM

The recent completion of several fungal genomes has provided a new way of studying the peroxisomal proteome. The two well-characterized peroxisome targeting signals, PTS1 and PTS2 (Neuberger et al., 2003; Maynard et al., 2004; Petriv et al., 2004), have been used to perform in silico searches of proteins that might be imported into the peroxisomal matrix (Emanuelsson et al., 2003; Galagan et al., 2005). Peroxisomes were previously thought to contain almost 50 enzymes, but current data suggest that the peroxisome matrix contains nearly 200 proteins in yeast and *Arabidopsis* (Kal et al., 2000; Kamada et al., 2003) and in *P. anserina* (V. Berteaux-Lecellier and O. Lespinet, personal communication). However, in vivo studies are still required to confirm the peroxisomal location of these newly identified proteins. The known peroxisomal enzymes are involved in various anabolic or catabolic pathways, some of which are ubiquitous, whereas others are more specific. In fact, peroxisomes are highly dynamic organelles whose content varies not only between organisms but also in function of the cell type. This is nicely illustrated in plants, where peroxisomes have been given different names based on their main activity in the cell; in germinating seeds, peroxisomes have been called glyoxysomes because of their involvement in the glyoxylate cycle, converting lipids into sucrose. At the start of photosynthesis, peroxisome metabolism plays an important role in photorespiration, and peroxisomes have thus been described as "leaf peroxisomes" (Hayashi and Nishimura, 2003). Here we mention the main metabolic peroxisomal functions of fungi, with emphasis on those that are particular to, and have been characterized more fully in, filamentous fungi. For detailed information on more general pathways, such as fatty acid β-oxidation and the glyoxylate cycle, the reader is advised to consult chapter 22.

Ubiquitous Functions

Ubiquitous functions for peroxisomes include the well-known peroxisomal fatty acid β-oxidation pathway. This pathway, together with the mitochondrial β-oxidation pathway, allows filamentous fungi to grow on various fatty acids (Maggio-Hall and Keller, 2004; Klose and Kronstad, 2006; Hynes et al., 2008; Boisnard et al., 2008). The peroxisomal pathway converts fatty acids into acetyl coenzyme A (acetyl-CoA), an important intermediary within cellular metabolism. Acetyl-CoA is required for several biosyntheses, and its ratio is tightly regulated (Schrader and Fahimi, 2008). Acetyl-CoA is further metabolized by the glyoxylate cycle within peroxisomes, to ultimately form carbohydrates (for further details, see chapter 22), which are used for various reactions in the cell. Moreover, mutant analyses in various fungus species suggest that the peroxisomal β-oxidation pathway is a source of acetyl-CoA in the synthesis of melanin (see "Peroxisomes in the Maturation and Germination of Fungal Spores" and "Peroxisomes in Predatory and Pathogenic Fungi" below; see also Kimura et al., 2001; Ramos-Pamplona and Naqvi, 2006; and Boisnard et al., 2008, and references therein). The peroxisomal β-oxidation of some fatty acids leads to pimelic acid formation, a biotin precursor (vitamin H or B7 [Ohsugi et al., 1988]). Consistent with this observation, peroxisomal biogenesis mutants altered for the peroxisomal β-oxidation pathway are auxotrophic for biotin (Hynes et al., 2008; V. Berteaux-Lecellier, A. Panvier-Adoutte, and M. Picard, unpublished data).

Beyond their role in β-oxidation, filamentous fungus peroxisomes are also involved in several other metabolic pathways, such as purine and amino acid metabolism. Some of these pathways require oxidase activities (acyl-CoA oxidase, D-amino-acid oxidase, urate oxidase, etc.); thus, normal peroxisomal metabolism constantly produces H_2O_2. Free radicals and derived species have important roles in signal transduction (Burdon, 1995; Lander, 1997; Bolwell, 1999; Aguirre et al., 2005; Jamet-Vierny et al., 2007); however, they are also highly corrosive. Therefore, peroxisomes contain antioxidant enzymes (like peroxidases and catalases) to regulate the level of ROS. Interestingly, a codistribution in peroxisomes of some H_2O_2 scavenger and producer enzymes is observed in filamentous fungi. The first step of peroxisomal fatty acid β-oxidation is usually performed by the acyl-CoA oxidase. This reaction produces enoyl-CoA and H_2O_2 (for further details see chapter 22); H_2O_2 is reduced to H_2O by catalase. Although both of these enzymes are located in peroxisomes in *A. nidulans* (Valenciano et al., 1996; Kawasaki and Aguirre, 2001), *N. crassa* peroxisomes contain an acyl-CoA dehydrogenase instead of acyl-CoA oxidase (and therefore do not produce H_2O_2 during fatty acid β-oxidation); no peroxisomal catalase has been detected (Thieringer and Kunau, 1991; Schliebs et al., 2006).

It is highly probable that peroxisome biogenesis defects disturb ROS homeostasis. Peroxisome impairment might have a significant impact on cell physiology, due to the

important role of free radicals in cell signaling. However, aside from the involvement of peroxisomal ROS in leaf senescence (del Rio et al., 1998), the mechanisms involved in the peroxisomal control of cell fate remain poorly described.

Specific Functions

Plants, yeasts, and filamentous fungi display a wide spectrum of peroxisomal activities, mainly due to the existence of peroxisome-specific functions.

Only a few filamentous fungi produce penicillin, one of the most used antibiotics: most notably, *A. nidulans* and *Penicillium chrysogenum* (Brakhage, 1997; van den Berg et al., 2007). The biosynthesis of this secondary metabolite is catalyzed by three enzymes and is a compartmentalized process (Brakhage, 1997; van der Lende et al., 2002). In particular, three pieces of evidence support the involvement of peroxisomes in penicillin biosynthesis. First, there is a clear positive correlation between penicillin production and the number of organelles per cell (Müller et al., 1991; Valenciano et al., 1998; Kiel et al., 2005). Second, the last step of penicillin biosynthesis (carried out by an acyltransferase, IAT) takes place in peroxisomes (Müller et al., 1991; Valenciano et al., 1998; van der Lende et al., 2002). Third, if the IAT putative peroxisomal targeting signal is removed, the enzyme remains in the cytosol and is still active in vitro. However, the mutant no longer produces penicillin (Muller et al., 1992). Interestingly, IAT is synthesized as a proenzyme, which has to be self-processed to be functional in vivo (Garcia-Estrada et al., 2008). This occurs inside peroxisomes, where both the pH value and the concentration of enzyme are presumably optimum for efficient self-processing. To further investigate the importance of peroxisomes in penicillin biosynthesis, studies relating to its production in various peroxisomal mutants should prove informative. However, attempts to obtain stable peroxisomal mutants in *P. chrysogenum* have been unsuccessful (Kiel et al., 2004) and true peroxisomal assembly mutants in *A. nidulans* have not been studied thus far for their capacity to produce penicillin (De Lucas et al., 1997; Hynes et al., 2008).

Methylotrophy is restricted to a limited number of microorganisms, and only a few yeast species are able to use methanol as a sole carbon and energy source (Yurimoto et al., 2005). In these yeasts, alcohol oxidase, which is localized in peroxisomes, catalyzes the first step of methanol assimilation and yields formaldehyde, a cytotoxic compound that can be either oxidized or assimilated (van der Klei et al., 2006). A set of genes involved in methanol utilization has recently been identified in the *P. anserina* genome (Espagne et al., 2008), and most of the genes have been shown to produce transcripts. If methylotrophic yeasts are shifted to a medium with methanol as the sole carbon source, a significant increase in peroxisomal size and number is observed (van der Klei et al., 2006), reflecting the intense methanol assimilation process occurring in the peroxisomal matrix. *P. anserina* growth on methanol results in fewer peroxisomes than on normal media; moreover, the peroxisomes are less dynamic and are mainly circular and clustered. In contrast to methylotrophic yeasts, these peroxisomes do not significantly increase in size. Thus, despite the presence of the enzymes required for methanol assimilation, overall findings indicate that *P. anserina* is not likely to be a true methylotroph. *P. anserina* grows on methanol-containing medium, but it appears to assimilate methanol poorly, and this leads to a highly vacuolated mycelium, strongly suggesting that the putative methanol assimilation pathways are rather involved in the detoxification of C1 compounds (Yurimoto et al., 2005).

PEROXISOMES IN THE REGULATION OF CELL MORPHOGENESIS AND DIFFERENTIATION

Filamentous fungi generate various cellular types to colonize different niches or to invade appropriate hosts. Also, these cell types are generated to ensure survival in hostile environments and to reproduce by either sexual or asexual processes (see chapters 33 to 36 of this book). Differentiation into various cell types implicates a rearrangement in the cell physiology. This is observed for peroxisomes, for which we observe variation in number and size during the course of fungal development. In growing hyphae, peroxisomes are mainly round or elongated and are regularly distributed throughout cells. In addition, peroxisome numbers can be significantly increased with substrates whose metabolism requires peroxisomal function, such as oleic acid (Valenciano et al., 1996). In *P. anserina*, the number, size, and localization of peroxisomes have been shown to vary largely during sexual development progression (Fig. 3). The size and distribution of peroxisomes in ascogonia (female sexually differentiated hyphae) are like those in growing hyphae. After fertilization, peroxisome numbers are particularly low in dikaryotic crozier cells (there are one to five), but peroxisome numbers increase after karyogamy. In the young growing asci (meiocytes), more than 100 peroxisomes can be observed, mostly concentrated at the tip of the cell. In mature asci, peroxisomes again appear to be randomly distributed and they do not seem to proliferate until ascospore formation, during which their numbers increase dramatically in the newly delimited ascospores (Berteaux-Lecellier et al., 1995; Peraza-Reyes et al., 2008a). Similarly, peroxisome numbers in the macroconidia of *Fusarium graminearum* are significantly greater than those observed in any vegetative developmental stage. This peroxisomal increase is correlated with high transcript levels for genes encoding many proteins implicated in peroxisome function, such as peroxisomal β-oxidation enzymes and peroxisome biogenesis and transport (Seong et al., 2008).

Fungal Dimorphism and Hyphal Morphology

Hyphal and mycelial morphology of most studied fungal mutants defective in peroxisomal function is not, or only slightly, affected under "nutrient-rich" growth conditions. In most cases, the observed deficiency is limited to a reduced growth rate; thus, studies implicating peroxisomes in the direct control of hyphal morphogenesis are limited. Nonetheless, peroxisomes play an important role in modulation of the morphogenic transitions that take place between the vegetative states of some dimorphic fungi, as well as in the establishment of their hyphal shape. One documented example is the dimorphic transition of the saprophytic yeast *Y. lipolytica*. This ascomycete displays various cell morphologies, ranging from yeast cells to septate hyphae, and some peroxins are required for the establishment of the hyphal morphology (Titorenko et al., 1997; Titorenko and Rachubinski, 1998). Moreover, the absence of a protein that controls peroxisome inheritance, Inp1p, induces filamentation under circumstances where normally a yeast form would prevail (Chang et al., 2007). The precise role of peroxisomes in the control of *Y. lipolytica* dimorphism has yet to be clearly established; however, they seem to participate in regulating the polarization of cells in an intricate manner with the endomembrane system and the secretory pathway of the cell.

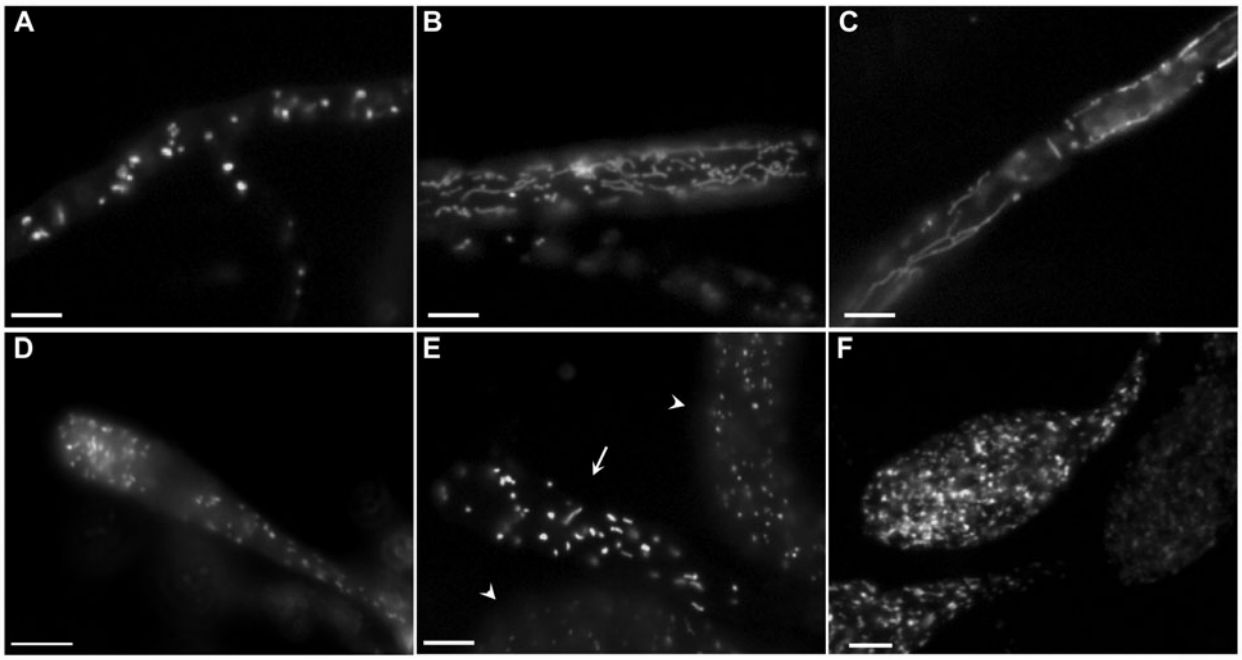

FIGURE 3 Peroxisome dynamics in filamentous fungi. Peroxisomes are highly dynamic organelles in filamentous fungi. They are typically round (A), but their number, form, and distribution can vary according to specific metabolical mycelial demands (A to C); during the formation of differentiated cells, such as during ascus development (panel D: observe the accumulation of peroxisomes at the growing apex of an ascus); between different cell types (panel E: compare the peroxisome shape in paraphysae [asexual cells present in fruiting bodies], shown by an arrow, and in asci, shown by arrowheads); and during spore formation (panel F: note the high peroxisome number resulting from the intense peroxisome proliferation occurring during ascospore formation). Images show peroxisomes of *P. anserina*. Peroxisomes were stained by the GFP-SKL reporter system (Ruprich-Robert et al., 2002); images were done in collaboration with D. Zickler. Scale bars, 5 μm.

Dimorphism in fungi may be related to the infective behavior of some pathogenic fungi. The maize pathogen *Ustilago maydis* normally adopts a unicellular yeast form when saprophytic and switches to the hyphal shape in order to infect its host. Fatty acids induce filamentation in in vitro cultures (Klose et al., 2004) and have a different effect on the growth pattern of induced mycelia. Interestingly, the peroxisomal multifunctional β-oxidation enzyme (Mfe2) of this fungus is required for the dimorphic transition induced by some but not all fatty acids. Moreover, in some situations in which the absence of Mfe2 does not impede filamentation, it reduces the branching of hyphae and, *in planta*, the infective hyphae of the *mfe2* mutants are virtually unbranched. These observations suggest that specific modified fatty acids, whose formation depends on the peroxisomal β-oxidation pathway, promote the dimorphic transition of this fungus and modulate its mycelial morphogenesis (Klose and Kronstad, 2006).

Asexual Cell Differentiation Processes

Developmental processes have energetic and metabolic demands different from those of vegetative growth and commonly require particular regulatory molecules. In fungi, some of these molecules can be in the form of fatty acid-derived secondary metabolites (Tsitsigiannis and Keller, 2007) or ROS (Aguirre et al., 2005), which could conceivably be formed or modified in peroxisomes. Similarly, peroxisome metabolism may contribute to the generation of key intermediary metabolites such as acetyl-CoA for energetic and biosynthetic purposes.

Conidium formation in *M. grisea* is drastically affected if peroxisome formation is defective. In this fungus, formation of aerial hyphae and conidia is largely diminished in mutant strains in which *pex6* has been deleted (Wang et al., 2007). At least some of these deficiencies can be explained by the reduced capacity of the mutant strains to supply peroxisomal acetyl-CoA, as mutant strains defective for the *pth2* gene, coding for a peroxisomal carnitine acetyltransferase, also show decreased conidiogenesis (Bhambra et al., 2006). Nevertheless, the conidiation default observed in the latter strains occurred to a lower extent, indicating that peroxisomal function is required for additional tasks (Wang et al., 2007). In the closely related saprophytic fungus *N. crassa*, functional peroxisomes are also required during conidiogenesis; however, the outcome of the peroxisomal dysfunction appears to be different, as mutants defective for the docking peroxin PEX14 are unable to form macroconidia from aerial hyphae (Managadze et al., 2007). In contrast, conidiation of *Colletotrichum lagenarium*, another sordariomycete, is not affected by peroxisomal dysfunction. In this pathogen, the number of conidia formed in the absence of isocitrate lyase (Asakura et al., 2006) or in *pex6* mutants with defective

peroxisome biogenesis (Kimura et al., 2001) is not affected. This complexity reflects the multiple mechanisms that fungi have evolved to control conidiogenesis.

An absence of functional peroxisomes in eurotiomycete fungi also results in different conidiation phenotypes. *P. chrysogenum* strains defective for *pex5* do not produce conidia (Kiel et al., 2004), whereas in *A. nidulans* conidia are formed but in significantly reduced numbers. In fact, the conidiation phenotype in *A. nidulans* is common to several mutants in which import of PTS1 proteins is affected. The effect was much less drastic in the absence of the PTS2 import pathway, and conidiation occurred normally in the absence of PexK (Pex11) (Hynes et al., 2008).

Asexual spore formation in *A. nidulans* and *N. crassa* is unaffected in the absence of isocitrate lyase activity, similar to what has been observed in the basidiomycete *Coprinopsis cinerea* (Flavell and Fincham, 1968; King and Casselton, 1977); consequently, the conidiation defects of peroxisomal mutants are not the consequence of a disrupted glyoxylate cycle. These ascomycete developmental deficiencies may result from defective WB formation; however, loss of HEX-1 only slightly affects development in *N. crassa* (Jedd and Chua, 2000; Tenney et al., 2000) and *A. nidulans* (Hynes et al., 2008), and no conidiation defects were observed in *M. grisea* (Soundararajan et al., 2004). This indicates that an absence of WBs influences conidiogenesis, but it is not the main cause of developmental deficiencies in these fungi. Interestingly, loss of HEX-1 in *N. crassa* abolishes cyclic conidiation induced by oleic acid in a wild-type strain; therefore, WB-mediated septal plugging may be implicated in coordinating periodic conidiation, which is controlled by the circadian clock of this fungus (Managadze et al., 2007).

Development of *A. nidulans* is controlled by fatty acid-derived oxylipin factors. These secondary metabolites, termed Psi factors, are derivatives of oleic, linoleic, and linolenic acids, and the proportions of their differently hydroxylated forms influence the ratio between sexual and asexual development (reviewed by Tsitsigiannis and Keller, 2007). In this fungus, oleic acid reduces the conidiation of a wild-type strain and induces the formation of cleistothecia (sexual fruiting bodies). Interestingly, this cleistothecial induction was not observed in a *pexF* (*pex6*) mutant, which could indicate that the peroxisomal metabolism influences oxylipin Psi factor formation (Hynes et al., 2008).

Sexual Development

The sexual development of some fungi takes place in large multicellular fruiting bodies, leading to the coordinated differentiation of numerous cell types. The development of these structures frequently occurs when mycelia have exhausted external resources, such that the metabolism of the emerging fruiting bodies becomes largely dependent on the reserve compounds of preexisting vegetative cells (Poggeler et al., 2006; Wosten and Wessels, 2006; see also chapter 33). Peroxisomal metabolism may play a major role in channeling nutrients from preexisting to newly differentiated cells of the developing fruiting bodies. The regulation of glyoxylate cycle enzymes in some fungi is in agreement with this assumption. In the ectomycorrhizal fungus *Tuber borchii*, the transcripts of genes encoding isocitrate lyase and malate synthase are much more abundant in fruiting bodies than in the vegetative mycelium. Moreover, metabolic resources of this ascomycete are relocated during fruiting-body development, and the lipids present in the vegetative hyphae of unripe fruiting bodies are mobilized to ripe ascospores upon maturation (Abba et al., 2007). However, in the basidiomycetes *Fomitopsis palustris*, *C. cinerea*, and *Schizophyllum commune*, the glyoxylate cycle appears to be regulated in an opposite manner, and glycolysis, together with the tricarboxylic acid cycle, seems to be the major pathway sustaining fruiting-body development (Cotter et al., 1970; Schwalb, 1974; Moore and Ewaze, 1976; Yoon et al., 2002). It is important to note that the lifestyle and fruiting-body constitution in these fungi differ; thus, these differences must reflect the diverse ways that fungi have developed to sustain fruiting-body development. Differentiation processes in *U. maydis*, culminating in sexual development, take place inside fungus-induced plant tumors and lead to the formation of diploid teliospores. The development of these teliospores is significantly delayed in the absence of the β-oxidation multifunctional enzyme (*mfe2*). Development in *U. maydis* is tightly connected to the virulence process; thus, it is possible that this developmental delay also reflects the lower proliferative efficiency *in planta* of the mutant strains (Klose and Kronstad, 2006).

A. nidulans produces few small cleistothecia in homozygous crosses of strains defective for the PTS1 peroxisome matrix import pathway (Hynes et al., 2008). A similar phenotype is observed in *P. anserina* mutants lacking the PTS1 receptor. However, in *P. anserina*, but not in *A. nidulans*, the lack of PTS2 receptor PEX7 acts as a partial suppressor of the *pex5* phenotype, suggesting that it is more detrimental to mislocalize a subset of peroxisomal matrix proteins than the complete matrix proteome in *P. anserina*. *P. anserina pex5* mutants also display a maternal effect during sexual development; i.e., the defect is observable in homozygous or heterozygous crosses whenever the *pex5* mutant is used as a female partner (Bonnet et al., 2006). These observations suggest that the incorrect compartmentalization of peroxisomal proteins affects the development of the maternally derived enveloping tissues of the fruiting bodies. Interestingly, this phenotype is not the consequence of a defective fatty acid β-oxidation pathway (Boisnard et al., 2008). The absence of PEX14 in *N. crassa* suggests an additional earlier peroxisomal role in protoperithecia development, as mutant strains defective for this peroxin are female sterile (Managadze et al., 2007). Remarkably, this phenotype is not observed if *P. anserina* lacks the corresponding peroxin (Peraza-Reyes et al., 2008b), suggesting fundamental differences in the physiology of even closely related fungi.

In addition to the fruiting-body constitution, peroxisomes also participate in differentiation processes taking place in the fertile portion (*centrum*) of these structures. *P. anserina* strains lacking the PTS receptors PEX5 and PEX7 exhibit abnormal formation of asci, resulting in ascospores with uneven numbers of nuclei or spores with no nuclei. The extent of this defect is considerably less in the absence of PEX7 than PEX5; moreover, *Δpex5* mutant also displays a maternal effect (Bonnet et al., 2006). The second *centrum* developmental event in which peroxisomes are involved in *P. anserina* is the transition from the prekaryogamy mitotic phase to the karyogamy and meiotic phase. PEX2 was the first protein shown to be required for this transition. Indeed, the mutation in *pex2* was found fortuitously when searching in mutant strains defective for ascospore formation (Simonet and Zickler, 1972), exposing one of the first developmental roles for peroxisomes (Berteaux-Lecellier et al., 1995). The phenotype of the *pex2* mutant is observed only in homozygous crosses and is characterized by an extended proliferation of crozier dikaryotic cells in which karyogamy never takes place and, consequently, ascospores are never formed (the *pex2* mutant phenotype is illustrated

in Fig. 11D of chapter 33). Nuclear fusion itself is presumably not affected in this mutant, as in vegetative cells it is normal (Berteaux-Lecellier et al., 1995); thus, it is likely that a prekaryogamy event required for the initiation of meiosis is impaired in these mutants. Moreover, this peroxisomal involvement may be specific to the differentiation of crozier/ascus cells in filamentous fungi, as the yeast *Pichia pastoris pex2* mutant (Waterham et al., 1996) can undergo the entire sexual cycle and form ascospores. Interestingly, so far the only proteins that have been implicated in this meiotic progression are all members of the *P. anserina* peroxisome RING finger complex; the same phenotype was observed in the absence of the other two RING finger peroxins, PEX10 and PEX12, but not in the absence of PEX5, PEX7, or PEX14 or in strains lacking both PEX5 and PEX7 (Bonnet et al., 2006; Peraza-Reyes et al., 2008b).

Likewise, none of the reported *A. nidulans* peroxin mutants (the orthologs of *pex1*, *3*, *5*, *6*, *7*, *11*, and *13)* display a similar phenotype (Hynes et al., 2008). This introduces the possibility of a bifunctional role for the peroxisome RING finger complex; however, so far, it has not been possible to dissociate discernible functions in peroxisome biogenesis and sexual development for the proteins of the RING finger complex (Peraza-Reyes et al., 2008b; our unpublished results). Moreover, there exist extragenic suppressors that partially restore the peroxisome assembly and developmental defects of cells defective for PEX2 (Ruprich-Robert et al., 2002; Boisnard et al., 2003; our unpublished data), indicating that in the presence of a defective RING finger complex there are alternative ways of restoring peroxisome biogenesis and sexual development. This also suggests that the role of the peroxisome RING finger complex in fungal development is not related to the classic peroxisome-matrix function/pathway and might reveal new unexpected functions for fungal peroxisomes.

Peroxisomes in the Maturation and Germination of Fungal Spores

The high number of peroxisomes in both *F. graminearum* macroconidia and *P. anserina* ascospores (Berteaux-Lecellier et al., 1995; Seong et al., 2008; see also under "Peroxisomes in the Regulation of Cell Morphogenesis and Differentiation" above for details) suggests a specific involvement of peroxisomes during spore maturation and germination. Spore maturation usually requires the incorporation of pigments such as melanin in the cell wall to make it stronger (Gomez and Nosanchuk, 2003). In filamentous fungi, melanin is produced by the dihydroxynaphthalene melanin biosynthesis pathway, either from malonyl-CoA or acetyl-CoA precursors (Langfelder et al., 2003; Ramos-Pamplona and Naqvi, 2006; Coppin and Silar, 2007). *P. anserina* peroxisomal assembly mutants have, to a different extent, ascospores that are less pigmented than wild-type ascospores (Berteaux-Lecellier et al., 1995; Bonnet et al., 2006; Peraza-Reyes et al., 2008b; Boisnard et al., 2008). Similarly, in plant pathogenic fungi, melanization of another fungal structure, the appressorium, is defective in peroxisome biogenesis mutants (Ramos-Pamplona and Naqvi, 2006; Kimura et al., 2001). Therefore, impairment of the peroxisomal fatty acid β-oxidation pathway that produces acetyl-CoA might account for this altered melanization. In line with this hypothesis, defects in melanization have also been observed in mutants affected in the peroxisomal β-oxidation pathway (Wang et al., 2007; Boisnard et al., 2008; see also "Peroxisomes in Predatory and Pathogenic Fungi" below for details). However, in the maize pathogenic fungus *U. maydis*, the teliospores formed by an *mfe2* mutant only display a delayed melanization (Klose and Kronstad, 2006), suggesting the existence of alternative sources for melanin precursors. Interestingly, such metabolites might be obtained from the host plant.

Beyond their impairment in melanization, ascospores from all *P. anserina* peroxisomal assembly mutants, along with the *fox2* (*U. maydis mfe2* ortholog) mutant strains, display a low germination frequency, suggesting a link between either β-oxidation or ascospore pigmentation and the germination process (Berteaux-Lecellier et al., 1995; Bonnet et al., 2006; Peraza-Reyes et al., 2008b; Boisnard et al., 2008). In fact, when *fox2* mutant ascospores are directly ejected onto the germination medium, the germination rate almost reaches that of the wild type (Boisnard et al., 2008). Thus, the fragility of less-pigmented ascospores rather than defects in the peroxisomal β-oxidation pathway is responsible for this germination impairment, at least for *P. anserina fox2* mutants. In *U. maydis*, the germination rate of the teliospores formed by the *mfe2* mutant is not affected, even if their melanization is delayed (Klose and Kronstad, 2006). Moreover, *A. nidulans* peroxisome assembly mutant ascospores, for which no defect in melanization has been reported, germinate efficiently (Hynes et al., 2008). Altogether these data show that peroxisomal fatty acid metabolism is not the only energy source required for the initiation of germination.

Ascospore germination studies of *P. anserina* peroxisomal assembly mutants and *fox2* mutant suggest that peroxisomes may also become involved in germination. Here, the growth rate of the germinative hyphae is reduced and gives rise to a spindly mycelium (Berteaux-Lecellier et al., 1995; Bonnet et al., 2006; Boisnard et al., 2008). This phenotype disappears for *pex2* mutant ascospores if glucose is added to the germination medium (Berteaux-Lecellier et al., 1995). The glucose requirement during germination is in accordance with *F. graminearum* microarray studies that showed that some of the most up-regulated genes associated with spores and spore germination are those involved in carbohydrate utilization and gluconeogenesis (Seong et al., 2008). Moreover, a phenotype similar to that of *P. anserina* ascospores is observed during the germination of *Aspergillus* conidia; this phenotype is delayed in *A. nidulans pex* mutants (Hynes et al., 2008) or when *A. fumigatus* isocitrate lyase and malate synthase mutants germinate under conditions resembling those faced after phagocytosis (Olivas et al., 2008). Moreover, if glucose is added to the medium, the germination rate of *A. fumigatus* conidia almost reaches that of the wild type (Olivas et al., 2008). Together these data suggest that the peroxisomal β-oxidation pathway and the glyoxylate cycle are required for the step following germination initiation, probably because they provide acetyl-CoA and carbohydrates to generate glucose for the further biosynthesis of cellular materials. Furthermore, intense degradation of trehalose has been observed during germination of *A. nidulans* conidia (Fillinger et al., 2001). Together, these data support the notion that peroxisomes are required under particular circumstances and that other carbon sources, notably trehalose, are required during conidial germination.

The importance of the glyoxylate cycle in spore germination is underscored by the *N. crassa* isocitrate lyase mutant phenotype (Flavell and Fincham, 1968; Gainey et al., 1992). In the absence of this key enzyme of the glyoxylate cycle, sexual spores fail to germinate or do not develop further than the germ tube state; however, *N. crassa* isocitrate lyase mutant conidia have no germination defect (Flavell and Fincham, 1968; Armitt et al., 1976). Moreover, neither the loss of isocitrate lyase activity nor peroxisome assembly

defects in *A. nidulans* lead to a defect in ascospore or conidium viability (Armitt et al., 1976; Gainey et al., 1992; Hynes et al., 2008).

The overall findings indicate that the peroxisome metabolic requirements for spore maturation and germination not only vary between sexual and asexual spores but also vary among filamentous fungi. Their specific requirements for these processes probably rely on the availability of external metabolites, which in turn is highly dependent on the fungus life cycle.

PEROXISOMES IN PREDATORY AND PATHOGENIC FUNGI

Many fungi are pathogenic to plants or animals. These fungi usually develop specialized structures to parasitize their host. For example, *Arthrobotrys oligospora*, a nematophagous fungus, differentiates, in the predacious phase, into three-dimensional adhesive networks (trap cells) to capture living nematodes (Barron, 1977). Studies performed with this fungus and other predatory fungi have shown that unusual peroxisomes are implicated in their developmental programs (Veenhuis et al., 1984; Dijksterhuis et al., 1994). During the transition from the saprophytic to the predatory stages, several cellular transitions occur and two classes of peroxisomes are observed: classical peroxisomes, which are present in vegetative hyphae in limited numbers, and electron-dense bodies that proliferate in trap cells from the initiation of trap development to the penetration of the host tissue (Dijksterhuis et al., 1994). These organelles are probably peroxisomes, as they contain catalase and D-amino-acid oxidase activities; however, they also possess unique attributes. Indeed, these organelles, like peroxisomes, are formed from a specialized region of the ER, but the major pathway for peroxisome production in the cell, by division of preexisting peroxisomes, is never observed. In addition, these bodies are surrounded by a thicker membrane and their electron density is high. These organelles are transitory and disappear by autophagy during the first stages of nematode infection, as soon as the mycelium has penetrated the cuticle and begun colonization of the host (Veenhuis et al., 1989; Dijksterhuis et al., 1994). The results of these cytological studies present an initial confirmation of a link between peroxisomes and fungal development. These unusual peroxisomes presumably play a crucial role in the initial stages of nematode infection. However, due to an absence of further studies, such as those investigating peroxisomal mutant pathogenicity, their role remains poorly understood.

A link between pathogenic development and peroxisomal function is also observed in phytopathogenic fungi, in which strains mutated for genes encoding peroxisomal proteins are less pathogenic than the wild type. The reported data strongly suggest that peroxisomes are implicated in pathogenesis, by producing precursors of the gluconeogenesis pathway via the glyoxylate cycle. Peroxisomes can also produce precursors for cell wall or melanin biosynthesis (Thines et al., 2000; Kimura et al., 2001; Asakura et al., 2006).

Mechanisms for the penetration and colonization of the host plant are well documented in fungi, such as the rice pathogen *M. grisea* (Talbot, 2003; Caracuel-Rios and Talbot, 2007) and *C. lagenarium*, the causal agent of cucumber anthracnose (Agrios, 2004). To penetrate their host plant, these fungi develop a specialized infection structure called the appressorium (Emmett and Parbery, 1975). The appressorium is a heavily melanized, dome-shaped single-cell structure that accumulates glycerol to a very high concentration. This allows, by hydrostatic turgor pressure, the production of a substantial invasive force required to perforate the leaf cuticle (Howard et al., 1991). The differentiation of this structure is accompanied by a rapid mobilization of lipid bodies, which are broken down by cytosolic triacylglycerol lipases generating fatty acids and glycerol (Thines et al., 2000). In *M. grisea*, this lipolysis and the associated release of glycerol appear to be the major source of turgor pressure generation (Wang et al., 2007).

Fungal growth requires carbohydrates for the biosynthesis of complex structural polysaccharides, such as glucans and chitin, to synthesize the cell wall. In the absence of external carbohydrates (i.e., during the first stages of infection), the peroxisome glyoxylate cycle may compensate by synthesizing glucose from lipids, which may explain their role during infection. Consistent with this idea, *C. lagenarium* mutant strains defective for the enzyme isocitrate lyase show a decreased frequency of host lesions, probably the consequence of a defect in the cell wall. This weakness in the cell wall may account for an increased sensitivity to the host defense responses (Asakura et al., 2006). Studies of various pathogenic fungi have shown that the glyoxylate cycle is also required at the beginning of the infection state, presumably due to the lack of a carbon source in the host environment (Lorenz and Fink, 2001; Idnurm and Howlett, 2002; Wang et al., 2003). However, the phenotype associated with the interruption of this cycle, at least in *C. lagenarium*, is not as drastic as when peroxisome biogenesis is hampered, suggesting that peroxisomes might have another role during the pathogenic cycle.

Fatty acids that are liberated by lipolysis are metabolized by β-oxidation—mostly peroxisomal β-oxidation—in the appressorium, generating acetyl-CoA. *M. grisea* mutant strains with knockout mutations in the genes encoding the peroxisomal β-oxidation multifunctional enzyme (Δ*mfp1*) or the peroxisomal carnitine acetyltransferase (Δ*pth2*), which are unable to export acetyl-CoA from the peroxisome to the cytoplasm, had a significant reduction in their ability to penetrate the plant cuticle and to colonize the host tissue. In addition, in the maize pathogenic fungus *U. maydis*, which does not produce true melanized appressoria, the infectious hyphae of the *mfe2* mutant are defective for the peroxisomal β-oxidation multifunctional enzyme. This mutant has virtually no branches, and its infective mycelium is less extended than the one from the wild type (Klose and Kronstad, 2006). These overall data highlight the importance of the cellular concentration of acetyl-CoA, first produced by the peroxisomal fatty acid β-oxidation pathway. Acetyl-CoA must then be exported from peroxisomes to the cytosol and other cellular compartments, to be used as a substrate in several metabolic and biosynthetic pathways to contribute to the host infection process (Bhambra et al., 2006; Wang et al., 2007).

The loss of virulence observed in the *M. grisea* Δ*mfp1* and Δ*pth2* mutant strains is accompanied by a decrease in the appressorium melanization. As described above ("Peroxisomes in the Maturation and Germination of Fungal Spores"), the dihydroxynaphthalene melanin biosynthesis pathway requires cytosolic precursors such as malonyl- or acetyl-CoAs. Disappearance of melanin is observed in the *M. grisea pex6* knockout mutant (see below). In this mutant strain, some peroxisomal enzymatic pathways, like β-oxidation, might be impaired due to the mislocalization of most peroxisomal proteins in the cytosol (Wang et al., 2007); this may partially account for the altered melanization process. However, a decrease in melanization does not appear to be the primary

reason for the loss of pathogenicity in these mutants, because addition of a melanin biosynthesis intermediate failed to restore the formation of lesions *in planta* and appressorium turgor pressure generation is normal in the Δ*pth2* mutant (Bhambra et al., 2006).

The above findings indicate that the role of peroxisomes in the infection process is complex and probably not restricted to the β-oxidation and glyoxylate pathways. The developmental regulation of the peroxisome number and of their enzymatic activities in pathogenic fungi suggests an important role for peroxisomes in the infectious developmental process. Moreover, in addition to the weak colonization of host plant tissue, Δ*pex6* appressoria are formed abnormally and with a low frequency (Wang et al., 2007), suggesting that peroxisomes are required at various stages of appressorium development.

It has recently become clear that appressorium formation in *M. grisea* is the consequence of a developmental process linking cell cycle progression and autophagic cell death (Veneault-Fourrey et al., 2006; Veneault-Fourrey and Talbot, 2007). Indeed, the appressorium appears to require the recycling of components produced by autophagy of preexisting cells. Peroxisome-produced acetyl-CoA could be a key component during this cell-remodeling process (Bhambra et al., 2006; Wang et al., 2007).

The multitude of functions fulfilled by the peroxisomal compartment is highlighted by the need for functional WBs (see "A Peroxisome-Related Organelle of Filamentous Fungi: the WB" above for details on WBs) in the formation of a functional appressorium in *M. grisea* (Soundararajan et al., 2004). Moreover, peroxisomes play also a part in spore formation and asexual developmental of this fungus (Wang et al., 2007). Thus, peroxisomes have diverse functions in the developmental process. The next challenge will be to better understand the link between peroxisomes and these developmental processes.

CONCLUDING REMARKS

To date, fungi have provided invaluable model organisms for studying peroxisome formation and function. Various yeasts have played a major role in the identification of several proteins implicated in peroxisome assembly and dynamics, as well as in the understanding of their function at the molecular and cellular levels. However, research into yeast developmental processes is limited; thus, filamentous fungi have been of great importance in revealing the developmental impact of peroxisomal function.

Peroxisomes provide fungi with a metabolic versatility that allows them to effectively colonize diverse habitats. In addition, they have been shown to be required for the execution of a number of developmental processes. Hence, the adaptability they provide is not limited to the capacity to effectively assimilate and metabolize diverse sources of carbon and nitrogen but also entails a metabolic relocation implicated in the modulation of cell fate. Furthermore, their involvement in developmental processes can vary between different fungal species, even when closely related phylogenetically. Thus, these fungal organelles appear to be adaptable and capable of adopting diverse roles with different physiological impacts. Interestingly, their plasticity extends far beyond the metabolic spectrum, as is nicely illustrated in fungi by the WB, a peroxisome-derived specialized organelle required for the filamentous lifestyle. In spite of the progress made in understanding peroxisome function in fungi, a detailed picture of how peroxisomes affect several other metabolic and developmental processes remains elusive. Further innovative approaches are required to fully understand the function of this organelle in fungi.

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HYPHAL GROWTH IV

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The Cytoskeleton in Filamentous Fungi

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In eukaryotic cells, dynamic polymers such as microtubules and actin filaments are important cytoskeletal elements that not only function as tracks for intracellular transport but also generate forces to make cell polarization/motility, organelle positioning, mitosis, and cytokinesis possible. Genetic tractability, coupled with the development of advanced imaging techniques, has made fungi excellent model systems for studying the cytoskeletal machinery. Currently, much of our knowledge of the functions and regulation of the cytoskeleton in fungi has been generated by research on several genetic model systems, including both the budding and fission yeasts and several filamentous fungi. In this chapter, our discussions are focused on the microtubule and actin cytoskeletons of filamentous fungi, including the motor proteins that are integral to cytoskeletal function. Relevant results from yeasts are discussed to provide background and context. The classical literature on the electron microscopy and immunofluorescence microscopy of the fungal cytoskeleton is not reviewed here. This has been ably and comprehensively reviewed by Heath (Heath, 1994a, 1994b, 2000). The emphasis is instead on more recent data from live-cell imaging as well as genetic and molecular genetic studies.

Filamentous fungi grow almost exclusively by polarized extension of the hyphal tip (Harris and Momany, 2004). To maintain such a highly polarized growth pattern, not only do cell wall precursors need to be continuously transported to the hyphal tip, but multiple nuclei and other organelles also need to be positioned properly within the elongating hyphae. It has been shown that both the microtubule and the actin cytoskeletons play roles in polarized growth of hyphae, and how these cytoskeletal elements function to support hyphal growth and organelle distribution in elongated hyphae is a topic of great interest.

THE MICROTUBULE CYTOSKELETON

A typical microtubule is a hollow tube composed of 13 protofilaments. The microtubule's building blocks, α-tubulin and β-tubulin heterodimers, are arranged in a head-to-tail fashion in protofilaments, which gives the microtubule its inherent polarity with β-tubulins terminal at the plus end and α-tubulins at the minus end (Nogales et al., 1999). It is worth pointing out that early experiments done by Morris and coworkers with the filamentous fungus *Aspergillus nidulans* led to the first discovery of genes for these tubulin subunits (Oakley, 2004). The microtubule plus ends are highly dynamic, with alternate growing and shrinking phases (Desai and Mitchison, 1997). Many proteins, such as the classic microtubule-associated proteins (MAPs) and microtubule plus end tracking proteins (+TIPs) are found to modulate the plus end dynamics in vivo (Carvalho et al., 2003; Howard and Hyman, 2003; Akhmanova and Hoogenraad, 2005). The minus ends are generally attached to microtubule-organizing centers (MTOCs).

In most fungi, the major MTOC is the spindle pole body (SPB), which is generally associated with, and often embedded in, the nuclear membrane (Jaspersen and Winey, 2004). SPBs contain γ-tubulin, which was first discovered in *A. nidulans* and is required for nucleation of microtubule polymerization (Oakley and Oakley, 1989; Oakley et al., 1990, Wiese and Zheng, 2006). Results from mutational analysis of *A. nidulans* γ-tubulin suggest that it also carries out additional functions essential for mitotic regulation and the organization of cytoplasmic microtubules (Jung et al., 2001b; Prigozhina et al., 2004). In filamentous fungi such as *A. nidulans*, microtubules are arranged longitudinally within the hyphae and microtubule nucleation occurs predominantly at SPBs. Thus, between any two nuclei it is likely that there are microtubules with mixed polarity, but in a region between the first nucleus and the hyphal tip, all microtubules seem to extend from the SPB towards the hyphal tip, suggesting that the dynamic plus ends of microtubules are at the hyphal tip and that there is no major MTOC at the tip (Han et al., 2001; Horio and Oakley, 2005; Sampson and Heath, 2005). However, the existence of minor MTOCs in addition to the SPBs is not excluded (Konzack et al., 2005). In the dimorphic fungus *Ustilago maydis*, there are also nonnuclear MTOCs activated at different stages of the cell cycle (Straube et al., 2003), and in *Allomyces macrogynus* (Chytridiomycota), there is a prominent apical MTOC (McDaniel and Roberson, 1998). Recent advances

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in live-cell imaging have allowed microtubule dynamics to be observed in several other filamentous fungi, including *Neurospora crassa*, and the application of electron tomography may provide further insights into microtubule organization in various fungal species (Freitag et al., 2004; Czymmek et al., 2005; Mouriño-Pérez et al., 2006; Hohmann-Marriott et al., 2006; Uchida et al., 2008).

In filamentous fungi, the microtubule cytoskeleton is essential for nuclear division, nuclear distribution, and transport of other organelles (Oakley and Morris, 1980, 1981; McDaniel and Roberson, 2000; Xiang and Plamann, 2003; Xiang and Fischer, 2004; Steinberg, 2007a, 2007b). Microtubules form mitotic spindles that are essential for chromosomal segregation during nuclear division. Microtubules almost certainly serve as tracks for the movement of vesicles, organelles, and RNA particles in the cytoplasm, and microtubule motor proteins are important for these intracellular transporting events (Xiang and Plamann, 2003; Steinberg, 2007a; Wedlich-Soldner et al., 2002a, 2002b; Becht et al., 2006; Soldati and Schliwa, 2006). The function of microtubules in rapid hyphal tip growth is likely related to the long-distance transport of cell wall precursors and polarity factors to the hyphal tip region (Horio and Oakley, 2005; Taheri-Talesh et al., 2008; Seiler et al., 2006; Vogt and Seiler, 2008) and to the communication with the actin cytoskeleton for maintaining directional hyphal growth (Fischer et al., 2008a, 2008b). Microtubules are also required for localizations of proteins in the septation initiation network (Kim et al., 2006a). In addition, dynamic microtubules may interact with the cortex to provide pushing or pulling forces directly on nuclei for nuclear positioning, as in yeasts (Finley and Berman, 2005; Tran et al., 2001; Maddox et al., 1999; Yeh et al., 2000; Daga et al., 2006). For microtubules to fulfill their cellular functions, the proper organization of the network is critical, and this also depends on proteins including microtubule motor proteins (Steinberg, 2007b).

MICROTUBULE MOTOR PROTEINS

Cytoplasmic Dynein

Cytoplasmic dynein, a multisubunit complex, is a minus-end-directed microtubule motor that plays a variety of roles in mitosis and in transport of cargoes such as vesicles, organelles, proteins, and mRNAs. The organization of the motor is different from that of the other motor protein families, kinesins and myosins (Vale, 2003; Sakato and King, 2004; Roberts et al., 2009), but the yeast dynein is a processive motor (meaning that it takes consecutive steps along a microtubule without falling off the track) and takes mainly 8-nm steps just like a plus-end-directed conventional kinesin (Reck-Peterson et al., 2006). The dynein heavy chain, which is a huge protein with a mass of about 500 kDa, is responsible for ATP-dependent motility. It contains an N-terminal tail that binds to other subunits of the dynein complex and a C-terminal motor unit comprising six AAA (acronym for ATPase associated with cellular activities) domains and a microtubule-binding stalk located between AAA4 and AAA5 (Pfister et al., 2006). Fungal dynein heavy chains show significant sequence similarity with mammalian dynein heavy chains, but interestingly, fungal dynein heavy chains are shorter due to a truncation at the C terminus (Vallee and Hook, 2006). Another interesting phenomenon is that in the dimorphic fungus *U. maydis*, the dynein heavy chain is encoded by two separate genes and synthesized as two polypeptides with the splitting point before the microtubule-binding site (Straube et al., 2001). Many other proteins involved in the in vivo function of cytoplasmic dynein have been discovered. They include proteins in the cytoplasmic dynein complex (Pfister et al., 2006); its accessory complex, dynactin (Schroer, 2004); and homologs of LIS1 (a product of the human disease lissencephaly gene) (Morris et al., 1998; Vallee and Tsai, 2006).

In fungal hyphae, cytoplasmic dynein is important for nuclear distribution and the positioning of organelles including endosomes (Xiang and Plamann, 2003; Xiang and Fischer, 2004; Steinberg, 2007a; Finley et al., 2008). It also plays nonessential roles during mitosis in spindle assembly, accurate chromosomal segregation, and spindle elongation (Efimov and Morris, 1998; Inoue et al., 1998; Li et al., 2005b; Fink et al., 2006a). In addition, dynein in filamentous fungi also participates in organizing the microtubule network by regulating microtubule dynamics and by providing force for transporting microtubules (Han et al., 2001; Fink et al., 2006b; Steinberg, 2007b). Finally, it has been found in *A. nidulans* that dynein loss of function causes an abnormal positioning of septa (Liu and Morris, 2000).

Mutants in the cytoplasmic dynein pathway have been isolated in *A. nidulans* as *nud* (acronym for nuclear distribution) mutants and in *N. crassa* as *ropy* mutants that exhibit nuclear clustering and hyphal growth phenotypes (Morris et al., 1998; Xiang and Plamann, 2003). Cloning of the *nud* and *ropy* genes in *A. nidulans* and *N. crassa* identified many components of the cytoplasmic dynein complex and the dynactin complex. Several genes encoding novel proteins important for dynein function were first identified in filamentous fungi, including NUDF (a homolog of mammalian LIS1 involved in neuronal migration), NUDE/RO11 (homologs of mammalian NudE/Nde1 and Nudel/Ndel1), and NUDC (a homolog of mammalian NudC and NudCL) (Osmani et al., 1990; Xiang et al., 1995; Minke et al., 1999; Efimov and Morris, 2000; Aumais et al., 2001; Vallee and Tsai, 2006; Zhou et al., 2006). The discovery of these fungal proteins as components in the dynein pathway has a great impact on studies in higher eukaryotic systems, especially studies on neuronal migration (Morris et al., 1998; Tsai and Gleeson, 2005; Vallee and Tsai, 2006). It should be pointed out, however, that not all proteins in dynein and dynactin complexes are essential for dynein's function in nuclear distribution. For example, loss of a dynactin component, p25, does not cause a nuclear distribution defect in *N. crassa* but a defect in vesicle positioning (Lee et al., 2001). In addition, loss of the 8-kDa dynein light chain in *A. nidulans* affects dynein function only at high temperatures (Liu et al., 2003).

In filamentous fungi, dynein and its regulators accumulate at the dynamic microtubule plus ends (Fig. 1), and the accumulation of dynein at the plus end depends on dynactin and kinesin-1 (Han et al., 2001; Zhang et al., 2003; Lenz et al., 2006). A similar localization has also been observed in budding yeast, where dynein at the plus ends of astral microtubules may be delivered to the Num1-containing regions of the cell cortex, where it exerts force to move the mitotic spindle (Lee et al., 2003; Sheeman et al., 2003; Miller et al., 2006). Spindle movement in *A. nidulans* may use the same mechanism, because the Num1 homolog ApsA in *A. nidulans* is also a cortical protein and is important for spindle movement (Xiang and Fischer, 2004; Veith et al., 2005). In *U. maydis*, premitotic nuclear migration toward the bud is dynein dependent, and the force exerted by dynein on the spindle pole body may also be involved in breaking the nuclear envelope for open mitosis (Straube et al., 2005).

Whether plus end dynein functions in interphase nuclear movement towards the hyphal tip is a question that needs to be addressed (Xiang and Fischer, 2004). Dynein at the microtubule plus end is involved, possibly indirectly through other +TIPs, in promoting microtubule dynamics (Carminati and Stearns, 1997; Han et al., 2001; Carvalho et al., 2003). It is possible that microtubule dynamics facilitates the interaction between a microtubule end and the cell cortex for pulling and/or pushing the nuclei around and the less dynamic microtubules in the dynein mutants could be a problem for nuclear migration. This notion is supported by the observations that the microtubule-destabilizing drug benomyl suppresses the nuclear distribution defect in the dynein mutants in *A. nidulans* and does so more dramatically in *Ashbya gossypii* (Willins et al., 1995; Alberti-Segui et al., 2001). However, loss of other +TIPs, such as the CLIP-170 homolog CLIPA or the Dis1/XMAP215/Stu2 homolog ALPA in *A. nidulans*, also causes microtubules to be less dynamic but does not produce an obvious nud phenotype (Efimov et al., 2006; Enke et al., 2007). Thus, dynein in *A. nidulans* may affect interphase nuclear migration through other mechanisms. Recent data suggest that dynein regulators such as NUDF and NUDC may be present at the SPB (Helmstaedt et al., 2008), but it remains to be determined whether they regulate dynein function at SPB to influence nuclear migration.

In filamentous fungi, the localization of cytoplasmic dynein to the dynamic microtubule plus ends is important for its function in retrograde transport of cargoes, especially early endosomes, inside the elongated hyphae (Steinberg, 2007a, 2007b). This was first demonstrated in *U. maydis* hyphae, where plus-end dynein is important for endosome loading and their subsequent transport towards the microtubule minus end (Fig. 2) (Lenz et al., 2006). Recently, green fluorescent protein (GFP)-Rab5A-labeled early endosomes have also been shown to undergo dynein-dependent retrograde movement in *A. nidulans* (Abenza et al., 2009). Given that dynein-mediated retrograde transport is crucial for the integrity of many cells, especially neurons (Chevalier-Larsen and Holzbaur, 2006), it is important to dissect the general regulatory mechanisms involved, and filamentous fungi are excellent model systems for these studies.

Kinesins

The genomes of most filamentous fungi analyzed to date contain 10 different kinesin-coding genes, except for the *A. nidulans* genome, which contains 11 kinesin genes (Schoch et al., 2003; Xiang and Plamann, 2003; Rischitor et al., 2004; Schuchardt et al., 2005; Galagan et al., 2005; Wortman et al., 2009). These kinesins belong to the kinesin-1, kinesin-3, kinesin-4, kinesin-5, kinesin-6, kinesin-7, kinesin-8, and kinesin-14 families (Lawrence et al., 2004; Schoch et al., 2003; Xiang and Plamann, 2003; Rischitor et al., 2004; Schuchardt et al., 2005). Here the discussion is focused on several kinesins that have been studied in filamentous fungi.

Kinesin-1

Kinesin-1 is a conventional kinesin that forms dimers and moves toward the plus ends of microtubules (Vale, 2003; Schliwa and Woehlke, 2003). Fungal kinesin-1 proteins show interesting differences in composition, structure, and properties relative to homologs in higher eukaryotes, and the reader is referred to reviews discussing these topics (Xiang and Plamann, 2003; Adio et al., 2006a). Although not essential for viability, fungal kinesin-1 proteins support vesicle trafficking important for rapid hyphal tip extension

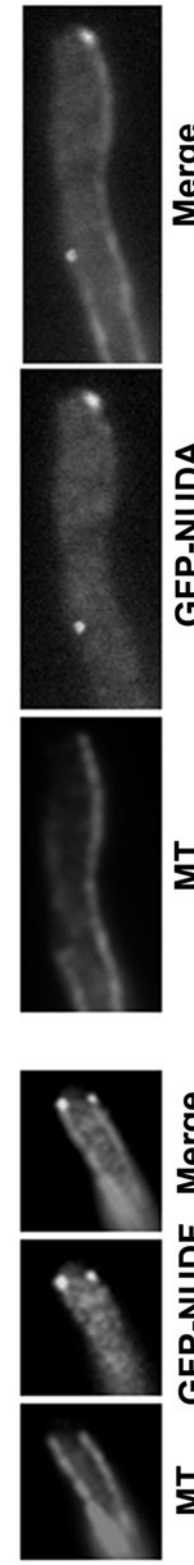

FIGURE 1 Microtubule plus end localization of GFP-labeled cytoplasmic dynein heavy chain (NUDA) and NUDF/LIS1. Microtubules (MT) are stained by an anti-α-tubulin antibody. This figure is a modified version of Fig. 2 from Han et al., 2001, with permission from Elsevier Ltd.

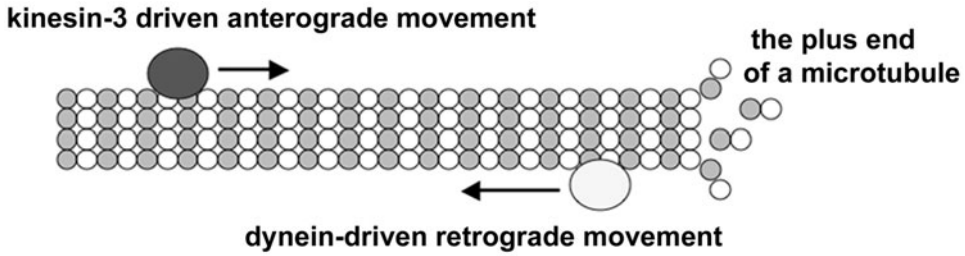

FIGURE 2 Diagram showing that early endosomes move bidirectionally along a microtubule. While the anterograde movement towards the microtubule plus end is driven by kinesin-3, the retrograde movement away from the plus end is driven by dynein (Lenz et al., 2006; Abenza et al., 2009; Zekert and Fischer, 2009).

(Xiang and Plamann, 2003; Requena et al., 2001; Schuchardt et al., 2005), and they may also play a role in microtubule organization (Straube et al., 2006). As a plus-end-directed motor, kinesin-1 is, surprisingly, also important for retrograde endosome movement from the hyphal tip (Steinberg, 2007a, 2007b; Abenza et al., 2009). This most likely results from a role for kinesin-1 in transporting cytoplasmic dynein to the plus end of microtubules, where dynein interacts with early endosomes for their retrograde transport (Zhang et al., 2003; Lenz et al., 2006; Steinberg 2007a, 2007b; Abenza et al., 2009). Finally, a kinesin-1 null mutant also exhibits a nuclear-distribution defect, although not as severe as that displayed by a dynein null mutant (Requena et al., 2001; Xiang and Fischer, 2004), and this may also be related to the role of kinesin-1 in the microtubule plus-end localization of dynein (Zhang et al., 2003).

Kinesin-3

Kinesin-3 proteins, members of the Unc104/KIF1 family, are mostly monomeric but can dimerize to become processive motors in the presence of high local protein concentration (Vale, 2003). Filamentous fungi including *A. nidulans* and *N. crassa* contain two members of the kinesin-3 family (Schoch et al., 2003; Xiang and Plamann, 2003; Rischitor et al., 2004). However, *U. maydis* contains a single kinesin-3, Kin3, which drives the anterograde transport of endosome-marker-containing vesicles toward the microtubule plus end close to the hyphal tip (Fig. 2) (Steinberg, 2007a). In *U. maydis*, kinesin-1 and kinesin-3 may both participate in transporting materials to the hyphal tip for hyphal growth, and this role becomes essential for polarized growth in the absence of an actin-based motor, myosin-V (Schuchardt et al., 2005). In *N. crassa*, the two kinesin-3 proteins, Nkin2 and Nkin3, play roles in the movement of mitochondria (Fuchs and Westermann, 2005). Specifically, Nkin2 is important for mitochondria to interact with microtubules in vitro and for mitochondrial movement in vivo. Nkin3, which is nonprocessive (Adio et al., 2006b), does not apparently associate with mitochondria in wild-type cells but can mediate mitochondrial motility in cells lacking Nkin2 (Fuchs and Westermann, 2005). Thus, the two kinesin-3 proteins may act redundantly to ensure proper mitochondrial transport. In *A. nidulans*, the UncA kinesin-3 transports vesicles toward the hyphal tip, and interestingly, it interacts preferentially with a special microtubule track apparently containing detyrosinated α-tubulin (Zekert and Fischer, 2009).

Kinesin-5

Kinesin-5 family members play roles in mitosis, and the founding member of this family is BimC in *A. nidulans* (Enos and Morris, 1990). At restrictive temperatures, the *bimC4* mutant exhibits a block in spindle-pole separation and forms monopolar rather than bipolar spindles (Morris, 1975; Enos and Morris, 1990). Interestingly, despite this block in spindle assembly, further rounds of DNA replication do not seem to be prevented, and thus, the *bimC4* mutant cell contains an abnormally large nucleus that is most likely polyploid (Enos and Morris, 1990). In *Saccharomyces cerevisiae*, the kinesin-5 proteins Cin8 and Kip1 also play important roles in spindle assembly mainly by pushing the spindle poles apart and maintaining a bipolar spindle (Saunders and Hoyt, 1992; Roof et al., 1992). Motors in the BimC (kinesin-5) family, such as Eg5 and Cin8, are homotetramers that most likely cross-bridge antiparallel microtubules and use their plus-end-directed motility to produce a force to move apart the spindle poles (Kapitein et al., 2005; Hildebrandt et al., 2006; Krzysiak and Gilbert, 2006). Remarkably, recent results have shown that Cin8 can also disassemble microtubules from the plus ends, which could be used as a mechanism for the length regulation of kinetochore microtubules to ensure proper chromosome congression (Gardner et al., 2008b).

Kinesin-7

Kinesin-7 proteins are plus-end-directed motors that are important for organizing the microtubule network in fungi. The *A. nidulans* KipA protein is involved in orienting microtubules so that growing microtubules merge at the center of fast-growing tips, which is important for directional hyphal growth (Konzack et al., 2005). It was observed in the *kipA* mutants that microtubule plus ends are able to reach the hyphal tip but exhibit continuous lateral movements. This may cause mislocalization of the vesicle supply center, or the Spitzenkörper (Reynaga-Peña, et al., 1997; Harris et al., 2005) near the center of the growing tip, and consequently a loss of hyphal directionality, which may explain why hyphae lacking KipA grow in curves (Konzack et al., 2005).

In both *S. cerevisiae* and *Schizosaccharomyces pombe*, the kinesin-7 proteins Kip2 and Tea2 transport the yeast homologs of CLIP-170 (the prototype of +TIPs), Bik1 and Tip1, respectively, to the microtubule plus end (Carvalho et al., 2004; Busch et al., 2004). Since Bik1 promotes microtubule growth in *S. cerevisiae*, the discovery that Kip2 transports Bik1 to the microtubule plus end provides the best explanation for the requirement of Kip2 for promoting microtubule growth (Carvalho et al., 2004). Both Kip2 and Bik1 are in the dynein pathway of spindle orientation and are important for dynein localization to the microtubule plus end (Sheeman et al., 2003; Carvalho et al.,

2004; Miller et al., 2006). *A. nidulans* KipA is also a +TIP, and a KipA rigor mutant protein (defined as one that cannot be detached from the microtubule track) decorates along microtubules, suggesting that KipA may move to the microtubule plus end by using its own motor activity (Konzack et al., 2005). However, KipA does not apparently play a critical role in dynein-mediated nuclear migration in *A. nidulans*. In *A. nidulans*, the CLIP-170 homolog CLIPA is also a +TIP and plays a redundant role with NUDE in targeting NUDF to the microtubule plus end (Efimov et al., 2006). KipA obviously enhances accumulation of CLIPA at the microtubule plus end at a higher temperature, consistent with a role for kinesin-7 in transporting CLIP-170 homologs in fungi (Carvalho et al., 2004; Busch et al., 2004). At a lower temperature, however, CLIPA accumulation at the plus end is independent of KipA (Efimov et al., 2006). It remains to be determined whether CLIPA may associate with the plus end, like CLIP-170, by binding to another +TIP, EB1 (end-binding protein 1), which exchanges rapidly at the plus end (Bieling et al., 2008; Dixit et al., 2009). How a temperature change affects the mechanisms of CLIPA localization also remains to be addressed.

In *S. pombe*, the CLIP-170 homolog Tip1 interacts with a cell end marker, Tea1, whose interaction with the formin For3p via Tea4 is important for localized actin assembly to ensure growth directionality (Martin et al., 2005b; Basu and Chang, 2007). The roles of the cell end marker proteins may be conserved between *A. nidulans* and *S. pombe*, as the cell end marker TeaA (Tea 1 homolog) in *A. nidulans* is also required for maintaining growth directionality (Takeshita et al., 2008). Although KipA does not seem to play a role in transporting TeaA to the microtubule plus end, it is important for proper localization of TeaA at the cortex, suggesting that other factors transported by KipA may be required for maintaining the proper localization of TeaA (Takeshita et al., 2008). A role for KipA in maintaining TeaA localization may explain why both *kipA* and *teaA* mutants exhibit curved hyphal growth, and it will be important to identify KipA cargoes in *A. nidulans* that are involved in maintaining TeaA localization and directional hyphal growth.

Kinesin-8

Kinesin-8 is a special family of kinesins that exhibit both plus-end-directed motility and kinesin-13-like depolymerase activity (Gupta et al., 2006; Varga et al., 2006). In *A. nidulans*, the kinesin-8 protein, KipB, is required for spindle positioning during mitosis, timely anaphase progression, and accurate chromosomal segregation (Rischitor et al., 2004). In both yeasts and mammalian cells, a role for kinesin-8 proteins in chromosome segregation has also been documented, which may be closely related to a function for kinesin-8 in modulating the kinetochore microtubules for bipolar chromosome attachment (West et al., 2002; Sanchez-Perez et al., 2005; Tytell and Sorger, 2006; Stumpff et al., 2008). In *A. nidulans*, KipB localizes to cytoplasmic, astral, and mitotic microtubules and moves along microtubules toward the plus ends (Rischitor et al., 2004). In *S. cerevisiae*, Kip3 functions in the Kar9 pathway of spindle orientation (Miller et al., 1998, 2006), and moves towards the microtubule plus ends, where it acts to prevent microtubule growth, thereby preventing the spindle orientated by the Kar9 pathway from being pushed back towards the mother cell (Gupta et al., 2006). *A. nidulans* KipB is important for mitotic spindle positioning, most likely also because of its function as a depolymerase at the microtubule plus end (Rischitor et al., 2004). KipB, however, is not required for interphase nuclear migration (Rischitor et al., 2004). Thus, dynein-dependent interphase nuclear migration may involve proteins other than those involved in spindle positioning such as KipB and ApsA (Rischitor et al., 2004; Veith et al., 2005; Xiang and Fischer, 2004).

Kinesin-14

Kinesin-14 proteins are in the Kar3/ncd family, contain a motor domain at their C terminus, and are minus-end-directed motors (Walker et al., 1990; Endow et al., 1994). In *A. nidulans*, the kinesin-14 family member is KlpA and deletion of its gene causes a suppression of the *bimC4* mutant phenotype, suggesting that KlpA and BimC play opposite roles in spindle assembly (O'Connell et al., 1993). This result is remarkably similar to that obtained from an independent study involving *S. cerevisiae*, whereby the loss of Kar3 suppresses the mitotic defect caused by loss of function of the kinesin-5 proteins Cin8 and Kip1 (Saunders and Hoyt, 1992). Thus, while kinesin-5 motors push the spindle poles apart, the kinesin-14 motors may counterbalance the force and pull the poles closer. Despite the role in antagonizing the kinesin-5 motors during spindle assembly, neither KlpA nor Kar3 is essential for mitosis. Nevertheless, several additional interesting functions of kinesin-14 proteins have been recently revealed in fungi. For example, a C-terminal deletion mutant of a kinesin-14, pkl1, is synthetically lethal with a γ-tubulin mutant in *S. pombe* (Paluh et al., 2000). In *A. nidulans*, a *klpA* null mutation, *klpA1*, significantly increases the cold sensitivity of three γ-tubulin mutant alleles. In synchronized cells, *klpA1* causes a transient inhibition of bipolar spindle formation and this effect is obviously exacerbated by the *mipAD123* γ-tubulin mutation, as many spindles never become bipolar in the double mutant. Thus, KlpA and γ-tubulin may play overlapping roles in the establishment of spindle bipolarity (Prigozhina et al., 2001).

Recent studies also indicate that the Kar3 protein, although originally thought of as being a minus end depolymerase (Endow et al., 1994), actually is targeted to the microtubule plus end by one of its light chains, Cik1, and acts as a depolymerase at the plus end (Sproul et al., 2005). In vivo results are consistent with this finding. For example, in mating yeast cells, Kar3 localizes to plus ends of overlapping microtubules, where it functions with Bik1 to drive nuclear congression by plus end capturing and depolymerization (Molk et al., 2006). Kar3 also plays a role in cross-linking spindle microtubules, which may be important for stabilizing anaphase spindles (Gardner et al., 2008a). In *S. pombe*, kinesin-14 klp2p also localizes to the microtubule plus end and organizes the bipolar arrays of microtubules with the microtubule bundler, ase1p (Janson et al., 2007).

THE ACTIN CYTOSKELETON

The actin cytoskeleton is essential for polarized growth of many cell types, including filamentous fungi (Seiler and Plamann, 2003; Harris and Momany, 2004; Fuchs et al., 2005). In filamentous fungi, the actin cytoskeleton and its myosin motors are important for the delivery of cell membrane and cell wall components to the growing hyphal tip and to the septum. The actin cytoskeleton may also participate in positioning mitochondria and Golgi equivalents in *A. nidulans* (Suelmann and Fischer, 2000; Hubbard and Kaminskyj, 2008). The organization of actin structures in filamentous fungi is probably similar to that in yeasts, where

three main types of actin structures can be found: actin patches, actin cables that emanate from the polarized growth sites (although actin cables are not as obvious as in yeasts), and septum-associated actin rings (Moseley and Goode, 2006; Harris et al., 1994; Torralba et al., 1998; Upadhyay and Shaw, 2008).

Actin cable formation in both budding and fission yeasts requires formin proteins that nucleate actin polymerization and are critical for actin cable elongation at the barbed ends that connect to the bud tip (Feierbach and Chang, 2001; Evangelista et al., 2002; Sagot et al., 2002a, 2002b; Pruyne et al., 2002; Moseley and Goode, 2006). Actin cables are most likely made of bundles of short filaments in both yeasts, and formin-mediated subunit insertion between the barbed end and the cell cortex may push other filaments in the bundle to move in a retrograde direction (Kamasaki et al., 2005; Martin and Chang, 2006; Buttery et al., 2007). The functional significance of retrograde actin flow is not clear, and whether this happens in filamentous fungi remains to be determined. It is interesting that in both yeasts, formins associated with the cell cortex (Bni1 in *S. cerevisiae* and For3 in *S. pombe*) may get released from the cortex and remain attached to the ends of the released actin filaments that undergo retrograde movements (Martin and Chang, 2006; Buttery et al., 2007).

Unlike the budding and fission yeasts, where two (Bni1 and Bnr1) and three (For3p, Cdc12p and Fus1) formins are found, respectively, filamentous fungi such as *A. nidulans* and *N. crassa* contain a single formin (Harris et al., 1997; Harris and Momany, 2004; Borkovich et al., 2004). The formin SepA in *A. nidulans* localizes to the hyphal tip and septa, where it organizes the formation of actin cables and septum actin rings, respectively (Sharpless and Harris, 2002). The fungus *A. gossypii*, which grows as filamentous hyphae but is evolutionarily close to *S. cerevisiae*, contains three formins, AgBnr1, AgBnr2, and AgBni1, but only AgBni1 is essential for hyphal growth. AgBni1 locates to hyphal tips and is required for forming actin cables for polarized transport of secretory vesicles (Schmitz et al., 2006). AgBni1 is similar to some other formins in that it may also be activated by Rho-family GTPases to release a self-folding-based autoinhibition, and a constitutively active form of AgBni1 changes the hyphal growth pattern in an interesting way. In cells expressing the active form of AgBni1, symmetric divisions of hyphal tips (tip splitting) can often be observed in young hyphae, a phenomenon that can only be seen in more mature wild-type hyphae, which undergo much more rapid elongation (Schmitz et al., 2006). Thus, a fine regulation of formin activity is important for maintaining the normal hyphal growth pattern, and too much formin activity may drive the formation of a much more robust actin cable system that would deliver too much material to maintain the growth of a single hyphal tip (Schmitz et al., 2006).

Formins are components of the polarisome complex involved in polarized growth (Sheu et al., 1998; Moseley and Goode, 2006). In *A. nidulans*, homologs of polarisome components such as Spa2 and Bud6 are also present, but they may function at different sites. While the Spa2 homolog SpaA seems to function only at the hyphal tip, the Bud6 homolog is detected only at the septum (Virag and Harris, 2006). The Spa2 homologs in several other fungi have also been studied. Spa2 in filamentous fungi such as *A. gossypii* is not essential for polarity establishment but is required for maximal polar growth (Knechtle et al., 2003), but in *Candida albicans*, it is important for polarity establishment and maintenance (Zheng et al., 2003). In *U. maydis*, Spa2 is important for morphogenesis and localizes to the hyphal tip, and this localization seems to be influenced by the small GTPase Rac1 but not by the integrity of the microtubules and the actin cytoskeleton (Carbo and Perez-Martin, 2008). Finally, studies of *A. gossypii* suggest that Spa2 and Bni1 but not Bud6 may be associated with Spitzenkörper, the vesicle supply center, during fast growth of hyphae (Kohli et al., 2008).

There may be interesting differences between filamentous fungi and yeasts in the temporal regulation of cytokinesis and polarized growth, two processes that require formin activity. Formins at the cytokinesis site are required not only for forming the actin ring (Tolliday et al., 2002) but also for the assembly of actin cables that can be used for the delivery of membrane and wall components for septation (Moseley and Goode, 2006). In *S. cerevisiae*, polarized bud growth occurs only early in the cell cycle, and the formin Bni1 and other polarisome components that were initially localized to the bud tip are targeted later during the cell cycle to the bud neck for cytokinesis (Moseley and Goode, 2006). In contrast, polarized hyphal growth in filamentous fungi occurs continuously regardless of cell cycle stages (Riquelme et al., 2003; Horio and Oakley, 2005), and SepA localizes to the hyphal tip and to the septum simultaneously (Sharpless and Harris, 2002). It is possible that subtle differences in formin regulation may have evolved to account for this difference. In addition, *C. albicans*, in which the yeast-hyphal transition occurs, may be well suited for studying how formins are regulated in these two different stages (Martin et al., 2005a; Li et al., 2005a; Rida et al., 2006).

The actin-patch-associated Arp2/3 complex is critical for formation of branched actin filaments (Pollard, 2007). Arp2/3 and its activators are conserved in filamentous fungi, but their functions require more study (Harris and Momany, 2004; Borkovich et al., 2004). In budding and fission yeasts, Arp2/3 and their activators are involved in the formation of endocytic actin patches that mediate endocytosis (Kaksonen et al., 2003; Galletta et al., 2008). The endocytic internalization machinery in *A. nidulans* appears to be similar to that in the budding yeast (Araujo-Bazán et al., 2008). Interestingly, in *A. nidulans*, the actin patches and, by inference, the sites of endocytosis are located primarily in a collar ringing the tip region that is spatially coupled to the site of exocytosis (Fig. 3) (Araujo-Bazán et al., 2008; Taheri-Talesh et al., 2008; Upadhyay and Shaw, 2008). Whether the Arp2/3 complex and its activators are used for both endocytosis and exocytosis or whether only the formin-mediated actin cables are important for exocytosis will need to be clarified.

Other actin regulators such as capping proteins are also conserved. Capping proteins function to block the addition and dissociation of actin monomers to and from the barbed end (Cooper and Sept, 2008). While the capping proteins are also involved in Arp2/3-mediated endocytosis in the budding yeast (Kim et al., 2006b), their roles in filamentous fungi remain to be defined. One interesting point is that capping proteins are also components of the vertebrate dynactin complex in which they cap the barbed end of the actin-related protein 1 (Arp1) filament (Schroer, 2004), but they are not essential for the spindle orientation function of the yeast dynactin complex (Moore et al., 2008). Functions of capping proteins in filamentous fungi will need to be determined. This will be interesting especially since the Arp1 pointed-end proteins are essential for dynein function in filamentous fungi, but not in the budding yeast (Lee et al., 2001; Clark and Rose, 2006; Moore et al., 2008; Zhang et al., 2008). Finally, the actin-binding protein tropomyosin is

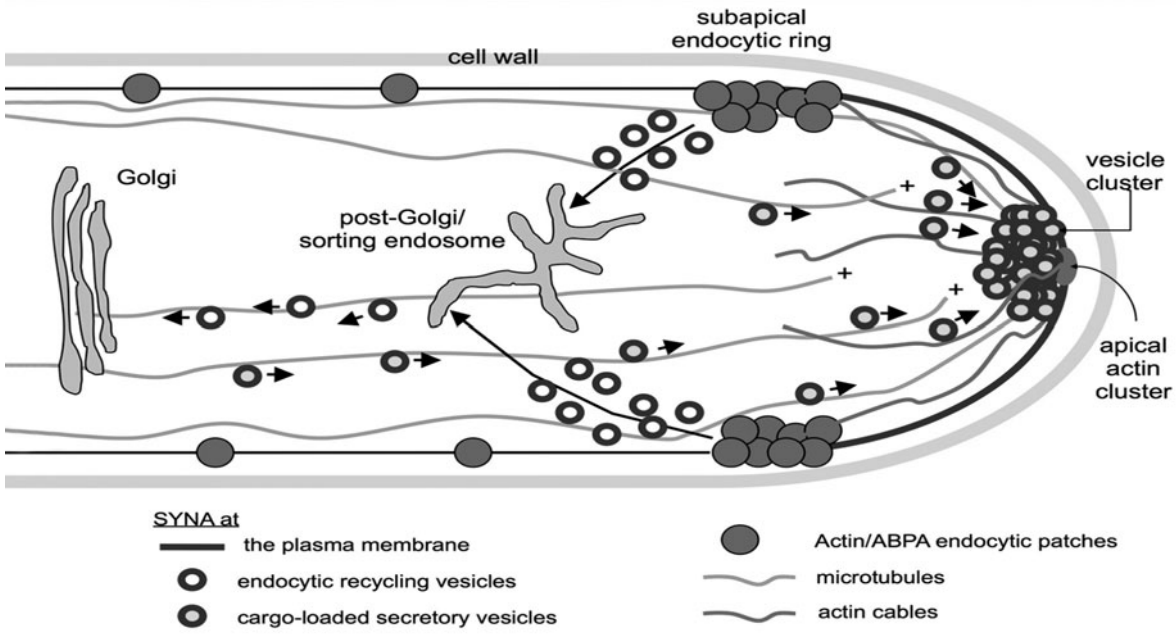

FIGURE 3 Model for tip growth in *A. nidulans*. The Spitzenkörper is not specifically labeled but includes (but is not necessarily limited to) the vesicle cluster and the apical actin cluster as well as the apical SSOA patch, SECC, and apical SEPA, which are not shown. Secretory vesicles containing components necessary for tip growth are transported toward the tip along microtubules powered by kinesin molecules (data not shown). The plus ends of microtubules are extremely dynamic. In some cases, they transiently contact the vesicle cluster. In such cases, the secretory vesicles could be transferred directly from microtubules to the cluster. In other cases, secretory vesicles presumably fall off the microtubule as the plus end disassembles, and they are transported to the vesicle cluster by myosin molecules (not shown) on actin cables. Vesicles fuse with the plasma membrane, releasing their contents, and the components of the membranes of the secretory vesicles (here represented by SYNA) become incorporated into the plasma membrane. As the tip grows, the ring of actin/ABPA endocytic patches moves forward, removing SYNA and other vesicle membrane components from the plasma membrane and incorporating them into endocytic vesicles for recycling. Although we do not have direct evidence, information from other systems and from M. Peñalva, J. Rodríguez, and J. Abenza (personal communication) indicates that these vesicles move to the post-Golgi sorting endosome by mechanisms that are not yet defined. From this compartment, SYNA-containing membranes move away from the tip on microtubules, powered by dynein, to be eventually incorporated into Golgi body-derived secretory vesicles containing cell wall biosynthetic enzymes and wall precursors. This figure and its legend are from Taheri-Talesh et al., 2008, with permission from the American Society of Cell Biology.

associated with the actin filaments in fungi and its acetylation may play a regulatory role in myosin-actin interaction (Pearson et al., 2004; Skoumpla et al., 2007).

ACTIN MOTORS: MYOSINS

Myosins are a diverse superfamily of actin motor proteins that play various cellular roles (Sellers, 2000). In filamentous fungi such as *A. nidulans* and *N. crassa*, four families of myosins have been found, including myosin-I, myosin-II, myosin-V, and the fungus-specific chitin synthases with myosin motor domains (Xiang and Plamann, 2003; Borkovich et al., 2004; Galagan et al., 2005; Wortman et al., 2009).

Myosin-I

Myosin-I, a single-headed motor, may participate in actin assembly through its interaction with the Arp2/3 complex (Higgs and Pollard, 2001). While a linker protein may be involved in the association of myosin-I with Arp2/3 in other cell types (Jung et al., 2001a), fungal myosin-Is are special in that their tails contain domains similar to that in the well-known Arp2/3 activator WASp, which may allow fungal myosin-Is to activate Arp2/3 directly (McGoldrick et al., 1995; Lechler et al., 2000; Evangelista et al., 2000; Lee et al., 2000; Higgs and Pollard, 2001). Consistent with this notion, synthetic lethality studies with yeasts have also suggested complementary functions of fungal myosin-Is and WASp homologs (Lechler et al., 2000; Evangelista et al., 2000; Lee et al., 2000).

The myosin-I from *A. nidulans*, MYOA, is required for initiating polarized growth, secretion, and septal wall formation (McGoldrick et al., 1995; Osherov et al., 1998). Consistent with these roles, wild-type GFP-MYOA localizes to the hyphal tip and septum (Yamashita et al., 2000). MYOA also plays a role in endocytosis, and this role was first revealed by an analysis of phosphorylation mutants of MYOA (Yamashita and May, 1998). Whether or not the motor activity of myosin-Is is involved in their cellular activities is an important question. In *A. nidulans*, a *myoA*

mutant that only contains 1% of its normal actin-activated ATPase activity and has no detectable in vitro motility can support polarized growth, suggesting that MYOA's function may be structural and not related to motility (Liu et al., 2001). Given the known interactions between fungal myosin-Is and Arp2/3, it is likely that MyoA is mainly required for organizing the actin patches in *A. nidulans* for polarized growth and endocytosis. It is important to note, however, that in *S. cerevisiae*, the myosin-I motor activity and actin nucleation are both required during endocytic internalization (Sun et al., 2006).

In *C. albicans*, the myosin-I CaMyo5 is also required for hyphal formation, which is similar to the situation in *A. nidulans* (Oberholzer et al., 2002). GFP-CaMyo5 co-localizes with cortical actin patches at both the bud and hyphal tip, and the null mutant forms random buds and also exhibits a depolarized distribution of the cortical actin patches. Interestingly, strains with specific mutations/deletions in CaMyo5 that exhibit depolarized actin patches are able to undergo hyphal growth, suggesting that CaMyo5 play distinct roles in actin patch distribution and polarized growth (Oberholzer et al., 2002, 2004).

Myosin-II

Myosin-II is the classic myosin, and its family members are involved in cytokinesis in both budding and fission yeasts (Moseley and Goode, 2006; Wu et al., 2006a). Myosin-II in *S. cerevisiae* also plays a role in retrograde actin flow in actin cables (Huckaba et al., 2006). Surprisingly, in the absence of a localized formin, yeast myosin-II localized at the bud neck may help to orientate actin cables for supporting polarized growth (Gao and Bretscher, 2009). The functions of myosin-IIs in filamentous fungi remain to be studied.

Myosin-V

Myosin-V is required for organelle transport in many organisms. In the budding yeast, where intracellular trafficking mostly depends on the actin cytoskeleton, one Myosin-V, Myo2p, drives transport of secretory vesicles, lysosomes, peroxisomes, and mitochondria, while the other myosin-V, Myo4p, has been implicated in the transport of endoplasmic reticulum and RNA-containing particles (Bretscher, 2003). In contrast to budding yeast, filamentous fungi use both microtubules and actin filaments for vesicle/organelle transport and for supporting polarized growth at the hyphal tip (Xiang and Plamann, 2003; Horio and Oakley, 2005; Fischer et al., 2008a, 2008b; Taheri-Talesh et al., 2008). The role of myosin-V in hyphal tip growth has been studied in *U. maydis*, which is a plant pathogen (Weber et al., 2003; Schuchardt et al., 2005). A GFP-Myosin-V fusion protein localizes to the hyphal apex as well as to the septa. However, myosin-V is not essential for hyphal tip growth or septation, but it is essential for conjugation tube formation. The defect in polarized hyphal growth is more pronounced after the mutant hyphae penetrate the plant epidermis (Weber et al., 2003). In the absence of kinesin-1 or kinesin-3, myosin-V becomes essential for hyphal growth (Schuchardt et al., 2005). In *S. pombe*, the myosin-V Myo52 has an important role in septum deposition and cell division (Mulvihill et al., 2006). However, myosin-V is not essential for septation in filamentous hyphae (Weber at al., 2003). Thus, filamentous fungi may utilize additional mechanisms for transporting cell wall materials to the septation site. Finally, Myo2p in *S. cerevisiae* moves the preanaphase mitotic spindle toward the bud neck by translocating the plus ends of astral microtubules along actin cables (Hwang et al., 2003; Miller et al., 2006). Whether this happens in filamentous fungi needs to be determined.

Chitin Synthases with Myosin Motor Domains

Remarkably, genes encoding chitin synthase with a myosin motor domain have been found in filamentous fungi (Fujiwara et al., 1997). In *A. nidulans*, CsmA and CsmB are class V and VI chitin synthases that contain a myosin motor domain. *csmA* or *csmB* single null mutants are viable, and the mutants show similar defects in polarized growth and cell wall integrity (Takeshita et al., 2002, 2005, 2006). However, the *csmA csmB* double null mutant is not viable, suggesting that the two proteins play important roles in polarized chitin synthesis (Takeshita et al., 2006). CsmA and CsmB both localize to actin-rich sites such as the hyphal tip and septa, and the myosin motor domains in both proteins are important for their interactions with actins, as evidenced by in vitro cosedimentation assays (Takeshita et al., 2005, 2006). Analyses of mutations in the myosin motor region in CsmA support the conclusion that this region is important for CsmA localization and function (Takeshita et al., 2005). In *U. maydis*, class V myosin chitin synthase (Mcs1) proteins localize to polar growth sites in both yeast-like cells and hyphae (Weber et al., 2006). The Mcs1 deletion mutant has no apparent hyphal growth defect, but mutant hyphae lose growth polarity after entering plants, and thus, this myosin chitin synthase is essential during early plant infection (Weber et al., 2006). In *Colletotrichum graminicola*, chitin synthase with a myosin motor domain is important for both polarized hyphal growth and plant pathogenicity (Werner et al., 2007).

INTERACTIONS BETWEEN THE ACTIN AND MICROTUBULE CYTOSKELETONS

Hyphal growth in filamentous fungi needs both microtubule and actin cytoskeletons, and thus, it would be important to understand how these two systems interact to coordinate vesicle transport towards the hyphal tip. It has recently emerged that the dynamic plus ends of microtubules play an important role in the interactions between these two systems, and +TIPs that associate with the plus ends are the critical factors (Carvalho et al., 2003; Akhmanova and Hoogenraad, 2005; Wu et al., 2006b; Basu and Chang, 2007; Fischer et al., 2008a, 2008b). Some of the +TIPs affect the directionality of growth in *S. pombe* and in *A. nidulans* (Basu and Chang, 2007; Fischer et al., 2008a, 2008b). For example, AlpA, the *A. nidulans* homolog of Dis1/XMAP215, and KipA, the *A. nidulans* homolog of Kip2/Tea2, accumulate at the microtubule plus ends and affect the growth directionality of the hyphae (Konzack et al., 2005; Enke et al., 2007). These +TIPs may affect the growth directionality by affecting the ability of plus ends to search for and capture targeting sites for the delivery of vesicles carrying membrane and wall materials. For example, a +TIP such as AlpA, which promotes the growth and dynamicity of microtubules, may facilitate such a delivery process (Enke et al., 2007).

A key discovery made with *S. pombe*, which has rod-shaped cells, is that the cell end protein Tea1 is delivered from the microtubule plus end to the cortex, where it interacts with the formin For3 to regulate actin assembly and polarized tip growth, and the Tea1-For3 interaction is mediated by Tea4, a protein that also gets delivered to the cell cortex by the microtubule plus end (Feierbach et al., 2004; Martin, 2005b). These results suggest that the microtubule

plus ends may direct the local assembly of actin cables. This idea is strongly reinforced by recent studies done in physically bent *S. pombe* cells, where microtubule plus ends contact cortical regions at the cell sides to induce formin-dependent polar growth at ectopic sites (Terenna et al., 2008; Minc et al., 2009). But surprisingly, polarized growth at these new sites depends on EB1, a +TIP, but not on the Tea1-Tea4 pathway (Minc et al., 2009). Recent studies with *A. nidulans* suggest that the cell end proteins are conserved in filamentous fungi (Takeshita et al., 2008; Fischer et al., 2008a, 2008b), and it is possible that general mechanisms of microtubule-plus-ends-directed local actin assembly may also be used in filamentous fungi. It is also important to note that filamentous hyphae undergo not only hyphal tip extension but also side branching, and how microtubule-actin interaction is involved in branching remains an interesting question.

How vesicles transported through microtubules get delivered to the actin cables is also an important question. In the budding yeast, myosin-V interacts with the microtubule plus end through EB1, and Kar9 mediates this interaction (Miller et al., 2006). While the interactions among these proteins in yeast are required for spindle positioning, it is remarkable that the connection between myosin-V and EB1 through another +TIP, melanophilin, has been found in vertebrate cells, and in this case, the interactions may be used for cargo delivery to the actin cytoskeleton from the microtubules (Wu et al., 2005, 2006b). Moreover, myosin-V may diffuse along microtubule tracks to obtain cargoes (Ali et al., 2007). It remains to be determined whether these and/or additional mechanisms operate in filamentous fungi and exactly how these events are regulated for directional transport of cellular materials during hyphal growth. Finally, it also remains to be determined whether in elongated hyphae of filamentous fungi, microtubule motors may transport factors that affect the actin cytoskeleton.

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The Cell Wall of Filamentous Fungi

NIR OSHEROV AND ODED YARDEN

The fungal cell wall performs multiple functions. It plays a significant role in development and integrity of the fundamental architecture required for survival and proliferation of the filamentous fungus. Also, the cell wall protects the fungus from abrasion, screens out poisons, and, by restricting inflation of the cytoplasm, allows the cell to become highly pressurized. It also serves as a scaffold for proteins that protect the inner polysaccharide layers and provide a dynamic interface with the surroundings. Fungal cell turgor ranges from 1 to 10 atm (Money, 1999). In comparison, the air in a car tire is compressed to a pressure of 2 atm. Generation of a cell wall and high turgor allow fungi to penetrate mineral and organic resources (Money, 1999). As the fungal cell wall is an essential structure of amazing complexity, it is not surprising that the fungal cell devotes a major fraction of its energetic output to its manufacture and upkeep. It accounts for 15 to 30% of the cellular dry weight (Nguyen et al., 1998). In the yeast *Saccharomyces cerevisiae* at least 20% of all genes are directly and indirectly involved in maintaining the cell wall (De Groot et al., 2001).

In addition to several comprehensive books covering this topic (Ruiz-Hererra, 1991; Gow and Gadd, 1995), several excellent reviews have been published in recent years describing the cell wall in yeast (Klis et al., 2006, 2007a; Ruiz-Herrera et al., 2006) and in filamentous fungi (Klis et al., 2007a; Latgé, 2007); its carbohydrate composition and biosynthesis (Bernard and Latgé, 2001; Adams, 2004; Latgé et al., 2005; Bowman and Free, 2006; Lesage and Bussey, 2006); cell wall proteins (CWPs) (De Groot et al., 2005; Dranginis et al., 2007; Richard and Plaine, 2007); the cell wall integrity pathway (Levin, 2005); and the cell wall as an antifungal target (Beauvais and Latgé, 2001; Selitrennikoff and Nakata, 2003; Nimrichter et al., 2005). This chapter provides a concise introductory overview describing the molecular composition and organization of the cell wall in the filamentous fungi. The major differences between the cell wall of the filamentous fungi and that of the better-studied yeast are stressed. Finally, take-home points regarding its dynamic structure and the many unanswered questions concerning its function are presented.

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THE MOLECULAR ORGANIZATION OF THE FUNGAL CELL WALL: AN OVERVIEW

To be strong yet pliant, the fungal cell wall is engineered as a composite material. It incorporates a mix of cross-linked fibers (the polysaccharides glucan and chitin) and matrix components (primarily proteins and mannans) (Fig. 1). It therefore resembles strong man-made composites, such as reinforced concrete (made of mesh and mortar) or carbon-fiber reinforced plastics (made of fibers such as fiberglass or para-aremid synthetic fiber [Kevlar], embedded in epoxy glue).

The main fibrous component of the cell wall is glucan, a polymer of glucose. Glucan can be found in long β-1,3 or short β-1,6-linked chains forming the main bulk (30 to 60%, dry weight) of the cell wall and in α-1,3 glucan. β-1,3 glucans have a coiled spring-like structure that confers elasticity and tensile strength to the cell wall. β-1,6 glucan acts as a flexible glue by forming covalent cross-links to β-1,3 glucan and chitin and to cell wall mannoproteins (Kollar et al., 1997; Shahinian and Bussey, 2000; Kapteyn et al., 2000; Lowman et al., 2003; Sugawara et al., 2004). α-1,3 glucan is amorphous in structure and forms an alkali-soluble cement within the β glucan and chitin fibrils (Beauvais et al., 2005; Grün et al., 2005). Chitin, the second cell wall fiber, is a long-chain β-1,4-linked polymer of *N*-acetylglucosamine, a derivative of glucose. It forms microfibrils that are stabilized by hydrogen bonds. Chitin provides tensile strength to the cell wall and composes ~2% of the total cell wall dry weight in yeast, and 10 to 15% in filamentous fungi (Roncero, 2002; Klis et al., 2002). The absence of chitin and most glucans from plant and mammalian species makes these components of the fungal cell wall potential and actual targets for antifungal drugs (Gooday, 1977; Beauvais and Latgé, 2001; Selitrennikoff and Nakata, 2003; Nimrichter et al., 2005; Latgé, 2007).

The matrix components gluing the fibers together are mainly mannans and proteins. Mannans are polymers of

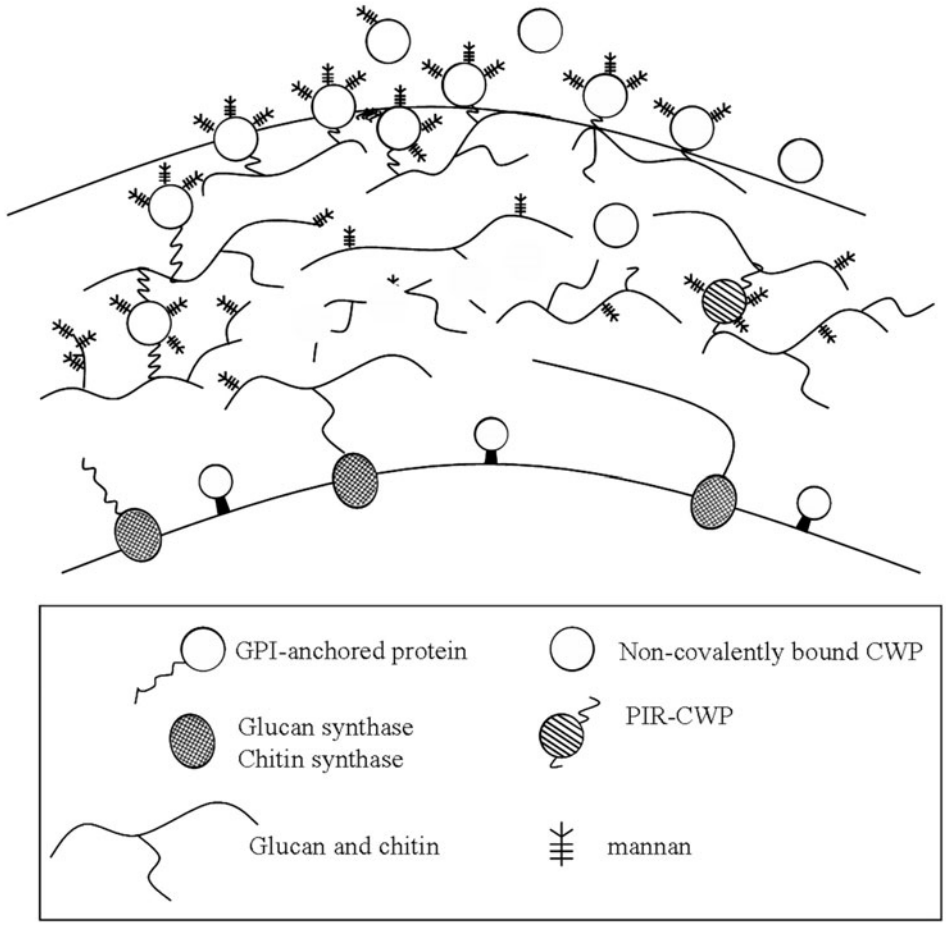

FIGURE 1 Structure and schematic representation of the architecture of a prototypical fungal cell wall. The outer layer contains high levels of different types of mannoproteins. The inner layer is made mainly of polysaccharides (β-glucans and chitin) and small amounts of proteins. The plasma membrane contains enzymes involved in β-glucan and chitin biosynthesis.

mannose and can be found as α-1,6 or α-1,2 or α-1,3/β-1,2 mannan chains either attached directly to glucans or covalently attached to proteins via asparagine (N-linked) or serine/threonine (O-linked) amino acid residues (Cutler, 2001; Shibata et al., 2007). They comprise 10 to 20% of the dry weight of the cell wall (Lesage and Bussey, 2006). The amount of glycoproteins in the cell wall is variable, between 10 and 40%, whereas the actual polypeptide content is about 4% (Klis et al., 2002; De Groot et al., 2007).

Cell wall composition has been studied most extensively in the yeasts *S. cerevisiae* (Kollar et al., 1997), *Schizosaccharomyces pombe* (Sugawara et al., 2004), and *Cryptococcus neoformans* (James et al., 1990; Vaishnav et al., 1998). In the filamentous fungi, detailed cell wall composition studies have been performed mainly with *Aspergillus fumigatus* (Fontaine et al., 2000), *Fusarium oxysporum* (Schoffelmeer et al., 1999), and *Neurospora crassa* (Bartnicki-Garcia, 1968), as well as in the dimorphic plant and human pathogens *Ustilago maydis* (Ruiz-Herrera et al., 1996) and *Candida albicans* (Lowman et al., 2003).

Cell wall composition varies extensively among the fungal orders. This variability is best viewed through the eyes of evolution; all fungal cell walls, including those of the distant chytrids, contain a branched polysaccharide of β-1,3/1,6 glucan that is linked to chitin via a β-1,4 linkage (Fig. 2A). This is likely the basic composition of an ancient ancestral fungal cell wall that has been further modified and decorated in the various fungal orders. For example, α-1,3 glucan appeared in the Ascomycetes and Basidiomycetes, and following their bifurcation, β-1,6 glucan was added to the former and xylose to the latter order (Ruiz-Herrera et al., 1996; Kollar et al., 1997; Vaishnav et al., 1998). Within the Ascomycetes, additional distinct differences have developed between the yeasts and filamentous fungi: the yeast *S. cerevisiae* contains β-1,6 glucan, whereas filamentous fungi such as *A. fumigatus* contain linear α-1,3/1,4 glucan and galactomannan with galactofuran side chains (Kollar et al., 1997; Fontaine et al., 2000) (Fig. 2B). The cell walls of the filamentous fungi contain higher levels of chitin than do those of yeasts (~15% versus 2 to 3%). The most likely reason for this difference is that being cylindrical and under high turgor pressure, the cell wall of filamentous fungi needs to increase its rigidity. Filamentous fungal cell walls also differ from those of the yeasts in their attachment to embedded proteins and mannans: in the yeast *S. cerevisiae* mannan chains are only found attached to CWPs, whereas in *A. fumigatus*, mannan chains are also found directly linked to glucans (Lesage and

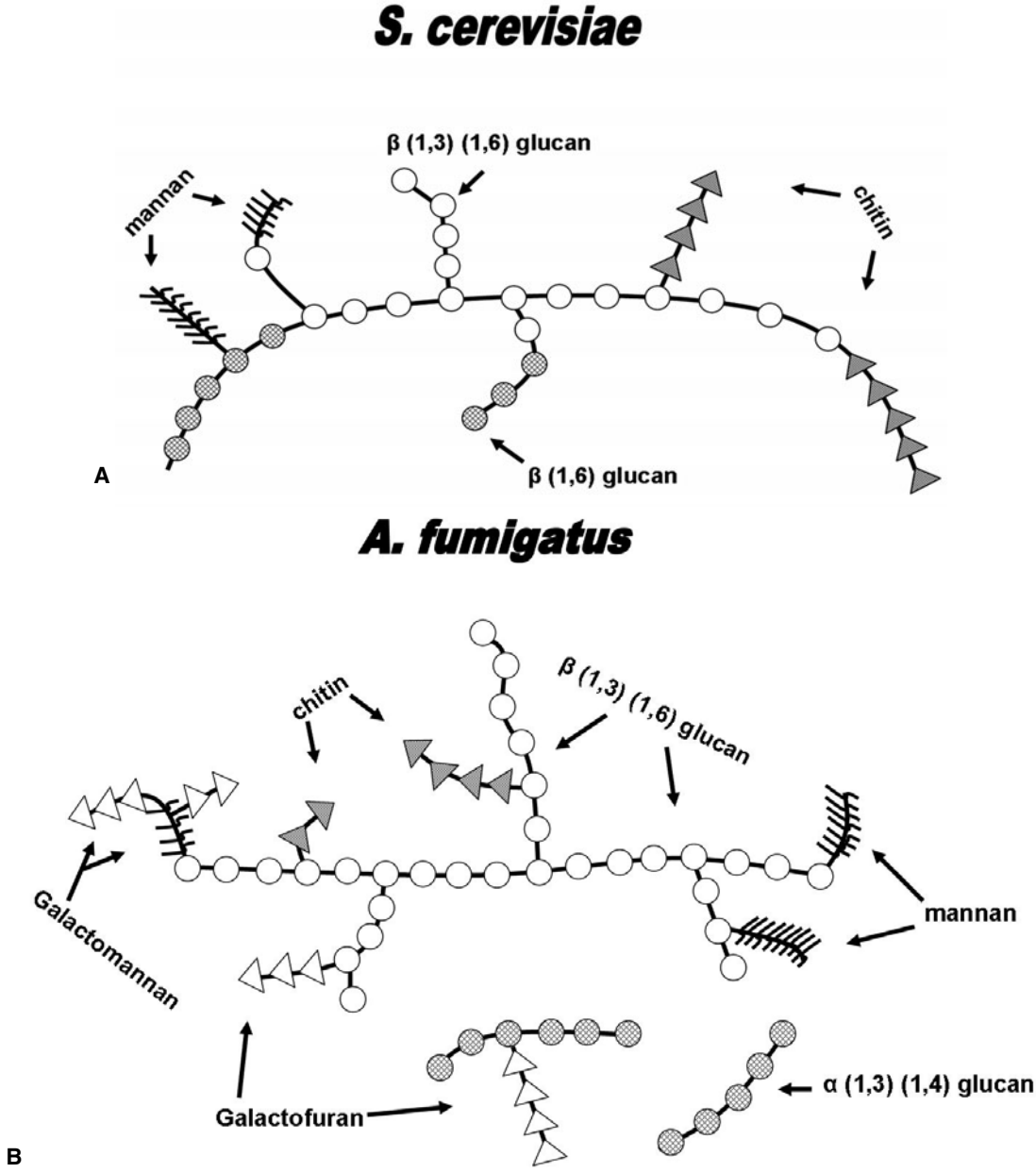

FIGURE 2 Comparison between the cell wall carbohydrate composition of a yeast (*S. cerevisiae*) (A) and a mold (*A. fumigatus*) (B). Both contain a branched polysaccharide of β-1,3/1,6 glucan that is linked to chitin via a β-1,4 linkage. However, only *S. cerevisiae* contains β-1,6 glucan, whereas *A. fumigatus* contains α-glucans and galactomannans that are not found in yeast.

Bussey, 2006; Latgé, 2007). Differences in the cell wall composition of yeast and filamentous fungi may have arisen because of differing evolutionary pressures; the filamentous fungal cell wall is adapted to extremely rapid deposition and growth at the hyphal tip and an ability to penetrate hard surfaces (Collinge and Trinci, 1974). In contrast, in yeast growth is largely isotropic and therefore slower, and it is usually confined to surfaces. This may explain why mutations in secretory and transport-related genes in yeast usually result in less severe phenotypic consequences than in filamentous fungi; the morphological complexity of the latter makes slight disturbances in homeostasis more obvious than they are in yeast (Whittaker et al., 1999; Seiler and Plamann, 2003).

MOLECULAR APPROACHES TO THE STUDY OF THE CELL WALL

The fungal cell wall in both yeasts and filamentous fungi is studied by various techniques that are addressed in this section. They include (i) the chemical analysis of carbohydrate composition; (ii) microscopic analysis (electron, light, and immunofluorescence); (iii) biochemical separation and identification of CWPs; (iv) use of cell wall biosynthesis

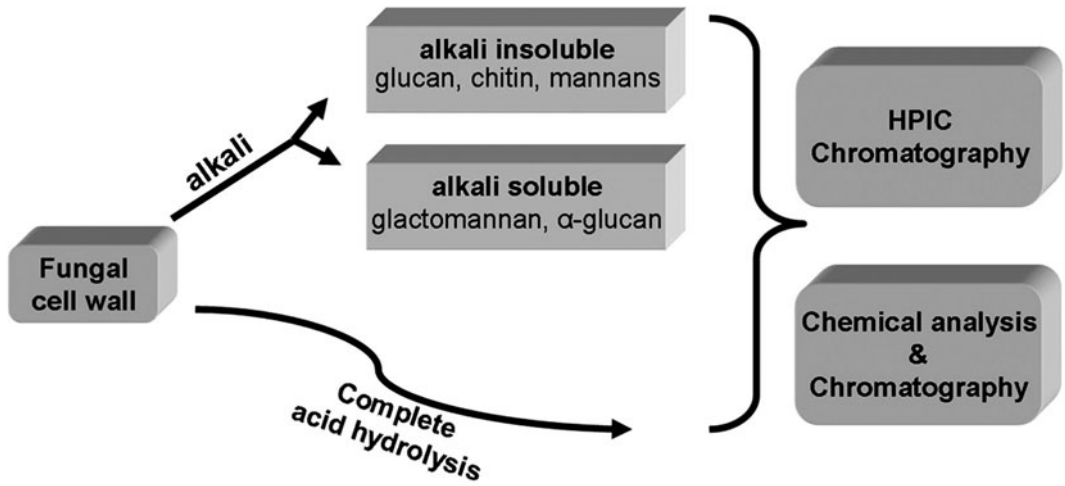

FIGURE 3 Schematic outlining the basic methods used to analyze the carbohydrate composition of the cell wall. Either the entire cell wall or alkali-digested soluble and insoluble fractions are completely digested by acid hydrolysis and analyzed by high-performance anion-exchange chromatography (HPIC) or chemical analysis and chromatography.

inhibitors and cell wall mutants; and (v) genomics, transcriptomics, and proteomics.

Chemical Analysis of Carbohydrate Composition

Chemical analysis has established the carbohydrate composition of the cell wall. The carbohydrate composition of the cell wall can be analyzed by two basic chemical methods (Fig. 3). The first is by complete acid hydrolysis of the carbohydrate chains into monomers (glucan to glucose, chitin to glucosamine, mannan to mannose, etc.) that are subsequently quantified by high-performance anion-exchange chromatography or enzymatic/spectrophotometric analysis (François, 2006). The advantage of this approach is that it is fast and can be adapted to perform high-throughput screening of mutants or to analyze changes in cell wall composition under various growth conditions (Ram et al., 1994; Dallies et al., 1998; Aguilar-Uscanga and François, 2003). The disadvantages are that it provides only the total levels of chitin, glucan, and mannan in the cell wall. Because complete carbohydrate hydrolysis is performed, it is impossible to identify the nature of the linkages between the different polymers and their subtypes.

The second, more traditional approach, involves solubilizing the cell wall in hot NaOH and separating the alkali-soluble and alkali-insoluble fractions. In *S. cerevisiae* the alkali-soluble fraction contains mainly mannans and some glucans. The alkali-insoluble fraction is then dissolved in cold acid to yield acid-insoluble (mainly β-1,3-glucan and chitin) and acid-soluble (β-1,3 glucan and β-1,6 glucan) fractions (Catley, 1988). Essentially the same method is used in *S. pombe* cell wall analysis (Pérez and Ribas, 2004). In *A. fumigatus* the alkali-insoluble fraction contains mostly β-1,3 glucan, chitin, some β-1,4 glucan, and galactomannan, and the soluble fraction contains α-1,3 glucan and some galactomannan (Bernard and Latgé, 2001).

Further fractionation is achieved by using specific endoglycosylhydrolases such as chitinase, β-1,3-glucanase, α-1,3-glucanase, and β-1,6-glucanase and by using acetolysis to internally cleave mannan chains (Bernard and Latgé, 2001; Magnelli et al., 2002; Pérez and Ribas, 2004; Magnelli et al., 2005). Finally, the carbohydrate linkages in the different fractions can be analyzed by specific carbohydrate chemistry methodologies (Kollar et al., 1997; Fontaine et al., 2000). The advantages of this approach are the ability to perform a complete carbohydrate analysis of the cell wall. The drawbacks include the time-consuming nature of the approach, the high degree of expertise needed to perform the analysis, and the lack of standardization in the field.

Microscopic Analysis

Microscopic analysis has established the layered structure of the cell wall and identified the spatial organization of its chemical and protein constituents. Traditionally, transmission electron microscopy or scanning electron microscopy (SEM) has been used to analyze the cell wall layers and their constitution (Osumi, 1998; Kaminskyj and Dahms, 2008). In most fungi, there are two layers; an electron-dense outer layer, and a light inner layer (Klis et al., 2007a). Numerous lines of evidence suggest that in yeasts such as *S. cerevisiae* and *C. albicans* and in filamentous fungi such as *N. crassa* and *F. oxysporum*, the dark outer layer is enriched in mannoproteins, whereas the inner translucent layer is composed primarily of glucan and chitin (De Groot et al., 2005). This can be shown by using proteases or glucanases to specifically remove the outer and inner layers of the cell wall, respectively (Kopecka et al., 1995; Koch and Rademacher, 1980; Zlotnik et al., 1984; Schoffelmeer et al., 1999; De Groot et al., 2005).

SEM has been used to monitor the construction of the cell wall following its removal by cell wall-degrading enzymes and has revealed that glucan is extruded from the plasma membrane as small particles that gradually coalesce into cross-linked fibrils (Osumi, 1998). Unlike transmission electron microscopy and SEM, atomic force microscopy, which uses a nanometer-wide tip to scan the specimen surface, can be performed on living cells. It has been used to probe (i) yeast cells during enzymatic cell wall removal, revealing a gradual roughening of the cell surface as mannans

were removed, exposing the underlying polysaccharide fibrils (Ahimou et al., 2003); and (ii) germinating *Aspergillus* spores, revealing the uncoating of the hydrophobic outer layer of proteins (collectively known as hydrophobins) that keep dormant spores dry (Ma et al., 2006).

Light microscopy and immunofluorescence (IF) are in widespread use for the analysis of CWPs and carbohydrates (Molina et al., 2000; De Groot et al., 2005). Antibodies used for IF interact specifically either with a CWP epitope or with a tagged epitope (i.e., *c*-Myc, 6HIS, FLAG, V5, etc.) engineered into the protein (Bates et al., 2007; Levdansky et al., 2007). Two main drawbacks to antibody-based IF are the need to fix the cells prior to staining and the limited penetration of the antibody inside the cell wall. Green fluorescent protein tagging of CWPs has also been used and in contrast to antibody-based methods can be used to visualize living cells. However, the large size of green fluorescent protein can cause artifacts, such as defective transport and mislocalization of the CWPs (Ram et al., 1998; Rodríguez-Peña et al., 2000; Pardini et al., 2006; Waterman et al., 2007). Various dyes and reagents are used for carbohydrate staining: β glucan stains with aniline blue (Shedletzky et al., 1997), and chitin stains with calcofluor white (CFW) or wheat germ agglutinin (Roncero et al., 1988). Carbohydrate-specific monoclonal antibodies specific towards mannan, galactomannan (Platelia Candida or Aspergillus antibody tests; Bio-Rad Laboratories), β-1,3 glucan, and α-1,3 glucan (Rappleye et al., 2007; Wheeler and Fink, 2006) have also become commercially available. However, a complete library of monoclonal antibodies specific to the different fungal glycosidic linkages needs to be developed. Such libraries, coupled with high-throughput methods, have revolutionized the study of plant cell walls (Moller et al., 2007).

Biochemical Separation

Biochemical separation has identified numerous CWPs. CWPs are relatively difficult to analyze (Yin et al., 2008). They are cross-linked to cell wall polysaccharides and are frequently glycosylated and of heterogenous size. CWP preparations are also easily contaminated with leaked cytosolic proteins (Klis et al., 2006).

Traditionally, the cell wall is first purified and then subjected to several steps of extraction: sodium dodecyl sulfate detergent washes to remove noncovalently bound proteins (e.g., secreted proteins), β-mercaptoethanol or preferably dithiothreitol extraction to remove additional noncovalently bound proteins and proteins covalently bound through cysteine residues, mild alkali extraction to remove glucan-bound proteins with internal repeats, and finally, hydrogen fluoride or glucanase treatment to extract glucan-bound glycosylphosphatidylinositol (GPI)-anchored proteins (Yin et al., 2008). Subsequently, the protein fractions are separated on a two-dimensional gel based on size and electrophoretic mobility and then fragmented proteolytically. The resulting peptides are analyzed by mass spectrometry (MS) to reveal their identities (Ebanks et al., 2006). However, this method is relatively hard to master technically, and because the CWPs are glycosylated to differing degrees, the individual protein spots are often fuzzy. Newer, more robust techniques include CWP extraction and proteolysis as described above, followed by liquid chromatography (instead of two-dimensional gel). Resolved peptides are fragmented by tandem MS (liquid chromatography/MS/MS), and the MS/MS spectra are searched against a fungal genome database to identify the corresponding CWPs (Yin et al., 2005). Recently, protein extraction has been simplified using "wall protein shaving." Intact fungal cells are incubated in the presence of proteases to digest accessible cell surface proteins. Because proteolytic enzymes are unlikely to permeate the plasma membrane of intact cells, this releases peptides that are associated specifically with the cell surface. Released peptides can be subsequently analyzed by MS, enabling the identification of cell surface proteins (Eigenheer et al., 2007).

Cell Wall Biosynthesis Inhibitors

Cell wall biosynthesis inhibitors are used to identify and characterize cell wall mutants. Testing for altered susceptibility to cell wall-damaging agents is commonly used to identify cell wall mutants in fungi. The most commonly used compounds are CFW and Congo red, which interfere with chitin synthesis; caspofungin and nikkomycin Z, which inhibit glucan and chitin synthesis, respectively; yeast killer toxin, which binds to a cell wall β-glucan receptor, forming lethal pores in the plasma membrane; Zymolyase, which degrades the β-1,3 glucan network; and less specific compounds such as sodium dodecyl sulfate, caffeine, and hygromycin B (Ram and Klis, 2006). Typically, mutants disturbed in the synthesis of glucan and chitin, mannosylation of mannoproteins, and GPI-anchor biosynthesis are hypersensitive to CFW and Congo red (Ram and Klis, 2006). In the filamentous fungi, *Aspergillus* mutants defective in glucan synthesis (Damveld et al., 2005a), CWP O-mannosylation (Shaw and Momany, 2002), and the cell wall stress response (Damveld et al., 2005b; Ronen et al., 2007) all exhibit CFW sensitivity.

CFW, yeast killer toxin, and caspofungin have also been used in mutant screens to identify genes that encode cell wall biosynthetic enzymes and structural CWPs involved in susceptibility (Brown et al., 1993; Bulawa et al., 1986; Ram et al., 1994; Lussier et al., 1997; Markovich et al., 2004; Hill et al., 2006). However, it is important to note that not all cell wall mutant phenotypes arise from mutation in genes directly involved in the cell wall. They can also arise because of defects in the cytoskeleton, in intracellular trafficking, and even in protein metabolism (Lussier et al., 1997; Pagé et al., 2003; Markovich et al., 2004). This is because the cell wall is a highly complex structure requiring the seamless integration of multiple cellular pathways and systems. Damage to any one of these can indirectly damage the cell wall.

Cell wall mutants can be analyzed using all of the above-described methods (microscopy, biochemistry, and inhibitors). For example, microscopic analysis of the *A. fumigatus* β-1,3 glucanosyltransferase Δ*gel2* deletion mutant revealed decreased melanization of the outer surface of conidia, and biochemical analysis of the cell wall showed an increase in chitin and a reduction in galactomannan (Mouyna et al., 2005).

Genomics and Transcriptomics

As described above, proteomic analysis has advanced considerably in recent years. More recently, genomics and transcriptomics approaches have emerged for analysis of the cell wall of filamentous fungi.

Genomic Analysis

To date, the genomes of 56 fungal species have been sequenced, including 35 species of dimorphic and filamentous fungi. It is now possible to identify approximately 50 to 100 putative CWPs in each of these species based on sequence

homology and conserved motifs, to reconstruct in silico cell wall carbohydrate biosynthesis pathways, and to select subsets of genes for further mutational analysis. Notable examples of genomic mining in filamentous fungi include the detection of all putative GPI-anchor-encoding genes in several species of filamentous fungi (De Groot et al., 2005) and the identification and analysis of repeat-containing putative CWPs in *A. fumigatus* (Levdansky et al., 2007).

Transcriptomics

The use of microarrays has revolutionized the analysis of the response of fungi to cell wall damage as well as the identification of genes transcribed during cell wall expansion/alteration during development. Notable examples of microarray studies involving the filamentous fungi include the transcriptional response of *C. albicans*, *A. fumigatus*, and *Aspergillus niger* to antifungal drugs (Liu et al., 2005; da Silva Ferreira et al., 2006; Meyer et al., 2007).

Efforts are under way to integrate different genome-wide analyses into one shared database. This type of data mega-clustering can reveal unexpected connections between biological processes and differentiate between novel and known effects in the analyzed experiments. One such database, with the acronym SIMBA (http://acgt.cs.tau.ac.il/simba/), is currently under development for the yeast *S. cerevisiae* (Tanay et al., 2005).

SYNTHESIS OF THE CELL WALL POLYSACCHARIDES

In filamentous fungi, growth and cell wall assembly occur mainly at hyphal apexes (Ruiz-Herrera, 1991). This is unlike the process observed in yeast, where extension occurs at bud tips, followed by intercalary growth. The carbohydrate polymers comprising the cell wall are synthesized de novo at hyphal tips by membrane-associated enzymes that are transported as inactive forms within vesicles to the tip region. Once incorporated into the plasma membrane, the active biosynthetic enzymes extrude the carbohydrate polymers through the membrane. This is followed by polymer cross-linking events and modifications performed by extracellular proteins. The mechanistic features of many aspects of polymer synthesis, extrusion, and assembly are still relatively uncharacterized.

Synthesis of Chitin, a Primary Cell Wall Component

The substrate for chitin synthase isozymes is UDP-*N*-acetylglucosamine (UDP-glcNAc), which is synthesized from fructose-6-phosphate by the Leloir pathway (Leloir and Cardini, 1953). In their pioneering work, Glaser and Brown (1957) used *N. crassa* when first describing cell-free chitin synthase activity. Since then, it has become evident that chitin synthases can be transported in vesicular organelles called chitosomes (Bartnicki-Garcia, 2006; Sietsma et al., 1996) and, based on in vitro experiments, some are activated by proteolysis (Bartnicki-Garcia, 2006).

The *N. crassa chs-1* gene was the first chitin synthase to be cloned from a filamentous fungus (Yarden and Yanofsky, 1991), following *CHS1* of *S. cerevisiae* (Bulawa et al., 1986). In time, it has become apparent that in contrast to the three chitin synthase genes found in yeasts (Cabib et al., 2001) filamentous fungi have multiple chitin synthase-encoding genes, the number of which can vary from 1 to over 20, and three classes of chitin synthases (designated III, V, and VI) are unique to filamentous fungi (Latgé, 2007; Choquer et al., 2004; Riquelme and Bartnicki-Garcia, 2008). A kinesin/myosin motor-like domain has been shown to be an integral part of one of one of these (class V), and the activity of these chitin synthases is associated with their binding to actin (Takeshita et al., 2005). Inactivation of some chitin synthase-encoding genes in various fungi results in no observable change in fungal phenotype, suggesting at least some redundancy in chitin synthase-encoding genes (Munro and Gow, 2001). Furthermore, the degree of overlapping functions of different chitin synthase classes in different fungal species remains unclear. The fact that the regulation of chitin synthases is complex and apparently involves various pathways (including the protein kinase C [PKC], osmotic stress, calcineurin and the cell wall integrity pathway) adds to the difficulty in dissecting the nature of chitin synthesis (Munro et al., 2007).

Synthesis of β-1,3 Glucan, the Major Cell Wall Component

The major polysaccharide core of the fungal cell wall is comprised of β-1,3 glucan. The substrate for β-1,3 glucan synthesis is UDP-glucose that is produced from glucose-6-phosphate by the activity of phosphoglucomutase and a uridylyltransferase. The synthesis of the β-1,3 glucan polymer is carried out by a glucan synthase protein complex, comprised of at least two proteins: the β-1,3 glucan synthase catalytic subunit (FKS) and a regulatory subunit (RHO) (Mazur and Baginsky, 1996; Qatoda et al., 1996). The number of genes encoding β-1,3 glucan synthases varies among different species, yet at least among filamentous ascomycetes analyzed so far, one FKS orthologue is apparently essential (Bernard and Latgé, 2001; Lesage and Bussey, 2006; Latgé, 2007). FKS (designated FK506-sensitive, as it was originally cloned by complementation of a mutant that was hypersensitive to the calcineurin inhibitor FK506 [Parent et al., 1993]) is a transmembrane protein that harbors a highly conserved hydrophilic signature motif (Douglas, 2001). The regulatory subunit is a Rho GTPase, whose activity is regulated by conformational changes that alter its GDP/GTP binding state as well as geranylgeranylation (required for transport and membrane attachment). The RHO subunit is maintained in an inactive form during vesicular transport and activated upon arrival at the plasma membrane (Abe et al., 2003).

There is evidence that in situ, β-1,3-linked glucans branch through β-1,6 linkages from the main β-1,3 glucan chain (Fontaine et al., 2000). β-1,6-Linked glucans play a pivotal role in cell wall assembly in yeast, where they interconnect all other wall components into a lattice (Klis et al., 2002). In sharp contrast, β-1,6-linked glucans are not found in a number of filamentous fungi, including *N. crassa* and *A. fumigatus*. Nonetheless, potential homologues of some yeast genes involved in β-1,6 glucan synthesis and transport have been discovered in some filamentous fungi (Borkovich et al., 2004), yet their roles have yet to be determined.

Regulation of Cell Wall Assembly

The synthesis of chitin and glucan is not sufficient for producing an intact and functional cell wall. Additional structural elements (notably galactomannans), once linked with glucan, contribute considerably to the integrity of the cell wall (Damveld et al., 2008). The biosynthesis of mannan and its galactofuran side chains has yet to be studied in depth (Bernard and Latgé, 2001), as is the function of various transglycosylases (glucanosyltransferases) involved in glucan modification and decoration. In addition to the biosynthetic and polymer-modifying enzymes, degradative

enzymes likely play important roles in the regulation and remodeling of the fungal cell wall. Spore germination, the formation of new hyphal branches, and conidial separation all require digestion of preexisting walls. Thus, specific members of the large families of chitinases and glucanases that have been identified in fungi are anticipated to play important roles in cell wall synthesis and remodeling (Adams, 2004; Seidl, 2008).

The orchestration of cell wall component biosynthesis and assembly involves both hierarchical and integrative modes of regulation. These include the transcriptional, translational, and posttranslational regulation of the gene and gene products involved in substrate synthesis, structural polymer biosynthesis, surface signaling, polarity, and secretion.

On the one hand, much progress has been made in the analysis of the basic physical components of the fungal cell wall. Yet many of the final stages of assembly and the dynamic changes that the cell wall undergoes have yet to be elucidated. Furthermore, even though links between cell wall biosynthesis and central signal perception and transduction pathways have been established (see "The Cell Wall Integrity Pathway" below), the comprehension of cell wall biosynthesis, at all its levels, is far from saturated.

COVALENTLY AND NONCOVALENTLY LINKED CWPs

CWPs Form the Interface between the Fungus and the Environment

CWPs perform diverse roles in the lives of fungi. They strengthen the polysaccharide cell wall polymers by cross-linking them, and by forming an outer coat, they protect them from hydrolytic degradation. In some cases, this protein coating masks the polysaccharides from recognition by the immune system (De Groot et al., 2005; Wheeler and Fink, 2006; Rubin-Bejerano et al., 2007). Also, CWPs mediate interactions with the environment, including adhesion and tissue invasion, biofilm formation, flocculation, and mating (Yin et al., 2008).

There are several categories of CWPs based on their mode of attachment to the cell wall (Fig. 1). CWPs are either covalently or noncovalently linked to cell wall polysaccharides. Covalently attached CWPs include (i) GPI-anchored CWPs, covalently linked to the β-1,3/1,6 glucan core through a trimmed form of their GPI anchor (Kollar et al., 1997) and (ii) alkali-sensitive linkage proteins including proteins with internal repeats directly linked to the β-1,3 glucan network through glutamine residues (Ecker et al., 2006). In both *S. cerevisiae* and *C. albicans*, they are proposed to function as cell wall cross-linkers and structural stabilizers (Martínez et al., 2004; Yin et al., 2005). Although members of this family have been identified in silico in several filamentous fungi including *N. crassa* and *M. grisea*, they have not been studied to date (De Groot et al., 2005). Noncovalently bound detergent-extractable CWPs often include proteins of cytosolic origin and secreted proteins. They are apparently bound by electrostatic interactions to the charged side chains of mannosylated proteins (De Groot et al., 2005).

GPI-Anchored CWPs

Most CWPs in yeast and filamentous fungi are GPI anchored. GPI-anchored proteins have been identified in silico by use of specially developed software (De Groot et al., 2003; Eisenhaber et al., 2004). These proteins contain a predicted N-terminal signal peptide and a C-terminal GPI anchor attachment site. Based on these analyses, there are predicted to be between 60 to 120 GPI-anchored-protein-encoding genes in the genomes of various ascomycetes (De Groot et al., 2003; Eisenhaber et al., 2004). GPI-anchored CWPs have also been identified recently in the basidiomycete mold *C. neoformans* (Eigenheer et al., 2007).

The GPI-anchored genes can be classified as those encoding (i) CWPs that are involved in cell wall biogenesis and remodeling including transglucosylases (Gas/Crh/Phr/GEL family), chitinases, and glycosyl hydrolases; (ii) CWPs with diverse enzymatic activities such as the lysophospholipases and proteases; (iii) adhesins (*ALS/FLO*) and hydrophobins; and (iv) with at least half of the genes in this category, proteins of unknown function, e.g., the Sps2/Ecm33 family, MP-1 family, CFEM-motif containing proteins and numerous conserved hypothetical genes (De Groot et al., 2005; Latgé, 2005).

Based on sequence homology, there is some functional overlap between GPI-anchored-protein-encoding genes in the genomes of ascomycete yeast and filamentous fungi, in particular for cell wall-modifying and -degrading enzymes. However, major differences exist even among closely related species. For example, 26% of *A. fumigatus* GPI-anchored-protein-encoding genes have no *A. nidulans* orthologues (our unpublished findings). This suggests that this class of fungal genes has rapidly diversified to meet the specific environmental needs of each species.

Only a small fraction of the GPI-anchored-protein-encoding genes in filamentous fungi have been studied by gene deletion (Latgé et al., 2005). In most cases, deletion results in no discernible phenotype or in subtle changes, such as altered germination rates, reduced adhesion, or changed susceptibility to cell wall-disrupting agents (for examples, see Bailey et al., 1996; Bernard et al., 2002; Damveld et al., 2005a; and Romano et al., 2006). There may be several reasons for these results, including (i) gene redundancy (especially for adhesins and cell wall-modifying enzymes that are found in large gene families) (Hoyer et al., 2007; Mouyna et al., 2005; Pardini et al., 2006); (ii) limited interaction of the gene product with specific external determinants (for example, the *C. albicans ALS3* adhesin specifically binds to buccal epithelial cells, but not to fibronectin-coated plastic [Zhao et al., 2004]); and (iii) expression under narrowly defined conditions (for example, *C. albicans* expresses a different set of GPI-CWPs in hyphal or yeast forms [De Groot et al., 2004]). In agreement with this last hypothesis, the actual number of GPI-anchored proteins isolated from the fungal cell wall under nutrient-rich conditions is far lower than the numbers predicted by in silico identification. Only 12 GPI-anchored proteins have been isolated from the cell wall of *S. cerevisiae* and *C. albicans*, and 8 such proteins have been isolated in *A. fumigatus* (Bruneau et al., 2001; De Groot et al., 2004; Yin et al., 2005).

Sequence Features Affecting Localization of Fungal GPI-Anchored Proteins

Fungal GPI-anchored proteins are either retained and localized to the plasma membrane or released and covalently bound to cell wall glucans. Early analysis of yeast suggested that retention of GPI-anchored proteins to the plasma membrane occurs primarily when two basic amino acids are found upstream of the GPI anchor site. However, results obtained with *C. albicans* suggest that the picture is not

clear-cut: the presence of long serine and threonine-rich regions, which are characteristic of many GPI proteins, favors targeting to the cell wall and may even override the plasma membrane-retaining effect of the two basic amino acids (Frieman and Cormack, 2004). Also, some of the GPI proteins containing membrane-retaining basic motifs have been nevertheless identified in the cell wall of *C. albicans* (De Groot et al., 2004). Therefore, the rules governing the differential localization of GPI proteins to the plasma membrane or cell wall have yet to be identified. This can be done by analyzing the localization patterns of a large number of these proteins. Additionally, the expression patterns of GPI proteins, under various growth conditions and at different time-points, need to be elucidated.

Several lines of evidence also suggest that at least in *A. fumigatus*, some GPI proteins are attached to the cell wall loosely, with no covalent linkages to β-glucan. The strongest line of evidence, based on MS analysis of the GPI-anchored acid phosphatase PhoAp, did not show that the C terminus of this protein was covalently bound to an oligosaccharide (Latgé et al., 2005). This might also be true for other fungi, since the identification of the chemical linkages between the protein and the polysaccharide, which remains the ultimate proof of such covalent linkages, has been assessed only for the *S. cerevisiae* GPI-anchored proteins Cwp2 and TIP1 (Van der Vaart et al., 1996; Fujii et al., 1999).

THE CELL WALL INTEGRITY PATHWAY

The cell wall serves as a major barrier between the organism and the environment. Thus, compromising the integrity of this structure leads to catastrophic consequences to the affected fungus. To orchestrate the establishment, growth, and maintenance of the fungal cell wall and to react to potential damage to the cell wall, a network of components involved in regulating cell wall integrity has evolved. This network, called the cell wall integrity (CWI) pathway, contains sensors, signal transducers, messenger molecules, regulators, and structural components.

In yeasts, members of the multiple Rho-type GTPase gene family regulate polarized growth by reorganization of the actin cytoskeleton and through signaling pathways that control the expression of cell wall biosynthetic genes. Rho1 is considered to be the master regulator of CWI in yeasts and has been the one most extensively studied, along with other components (reviewed by Levin, 2005). Rho proteins reside at the plasma membrane (this association is aided by the fact that they are C-terminally prenylated). Like other G proteins, Rho proteins cycle between the active GTP-bound state and the inactive GDP-bound state. The Rho protein cycle is regulated both by GTPase-activating proteins and guanosine nucleotide exchange factors acting in opposing directions. The Rho proteins receive their initial stimulus from transmembrane cell surface sensors, some of which are linked with additional signal transduction pathways. Thus, some components of the CWI pathway interface with Tor kinase signaling, Ca^{2+} signaling, phosphatidylinositide signaling, and sphingoid base signaling (Levin, 2005). Together, these components coordinate changes in the actin cytoskeleton, cell wall maintenance, expansion, and polarized secretion. Known effectors for Rho1 include PKC, β-1,3 glucan synthase, formins, transcription factors, and exocytosis components (reviewed by Levin, 2005).

Overall, the backbone of the CWI pathway in filamentous fungi (Fig. 4) appears to resemble that found in yeasts (Levin, 2005). Thus, Rho-type GTPases and PKC have been found to be involved in morphogenesis and cell wall (including septal) biosynthesis, remodeling, and fungal pathogenesis (Guest et al., 2004; Rasmussen and Glass, 2005; Martínez-Rocha et al., 2008; Teepe et al., 2007). Furthermore, the filamentous fungal CWI pathway has been found to activate chitin synthesis as well as genes encoding α-1,3 glucan synthases (Damveld et al., 2005b), via a conserved transcription factor (Damveld et al., 2005a). The latter does not occur in *S. cerevisiae* and *C. albicans*, as these yeasts lack the genes encoding the α-1,3 glucan synthases. The CWI pathway functions in concert with other sensing, transducing, and regulatory networks. This is exemplified in cases where the CWI pathway is activated following the activation of the calcineurin pathway or following the activation of the high-osmolarity mitogen-activated protein kinase pathway (in *S. cerevisiae*) or when cell wall inhibitory compounds are applied (respectively discussed by Harel et al., 2006; Bermejo et al., 2008; and Meyer et al., 2007). One method for the identification of additional CWI pathway components (such as the recently identified UDP-galactopyranose mutase) involves using reporter genes linked to cell wall stress-induced promoters (Damveld et al., 2008). *S. cerevisiae* clearly serves as an invaluable model for identification of CWI pathway components. Nonetheless, filamentous fungi do not necessarily have obvious homologues to all the identified yeast components and differences from the yeast model have been reported (Fujioka et al., 2007). Furthermore, the composition and morphological complexity of cell walls in filamentous fungi promise to yield novel regulatory and structural constituents of the CWI pathway. Examples of this complexity have already been demonstrated in cases where mitogen-activated protein kinase pathways act in both concerted and overlapping manners in the regulation of hyphal fusion and sexual reproduction, as well as in an interactive manner with additional pathways in the regulation of hyphal elongation/branching, as well as other developmental and metabolic pathways (Maerz et al., 2008; Park et al., 2008).

THE CELL WALL AS AN ANTIFUNGAL TARGET

The requirement of a functional cell wall for survival, growth, development, and pathogenicity of fungal species makes it an attractive target for antifungals. The fact that some of the constituents of the fungal cell wall are not present in potential hosts makes this target even more appealing on the basis of a desirable therapeutic index. As such, the fungal cell wall has been a constant focus of antifungal drug screening procedures. Compounds affecting a multitude of cellular processes can have various effects on the cell wall, yet only a few specifically affect cell wall biosynthetic components (Ruiz-Herrera, 1991). This section focuses on compounds affecting specific constituents of the fungal cell wall.

CHS Inhibitors

Chitin synthase (CHS) inhibition is mainly based on utilizing analogues of the CHS substrate UDP-*N*-acetylglucosamine. The two major groups of peptide nucleoside antibiotics used to inhibit CHS are polyoxins and nikkomycins (Dähn et al., 1976; Endo and Misato, 1969; Müller et al., 1981; Graybill et al., 1988). This group of competitive CHS inhibitors includes natural products of *Streptomyces* spp. and their derivatives that inhibit CHS in various fungi. As the active site of the membrane-associated CHSs faces the cytoplasm, the efficacy of the substrate ana-

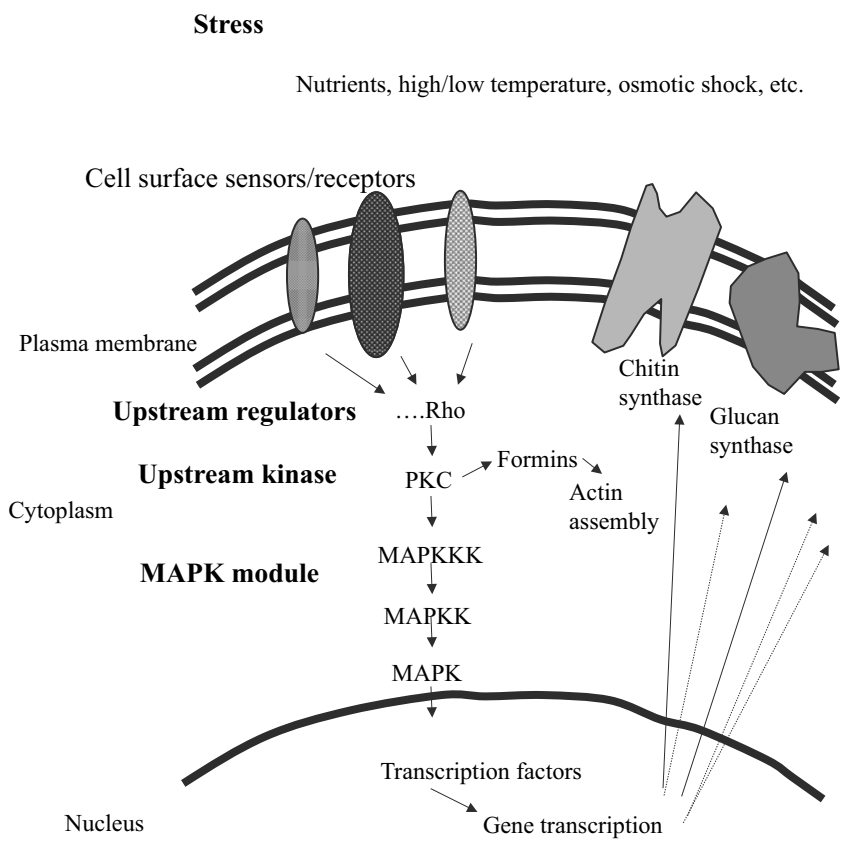

FIGURE 4 Outline of the fungal cell wall integrity pathway. Activation of the pathway involves perception of external signals (including stress- and nutrition-related cues) by membrane-associated sensors and signal transducers, intracellular transducers and messenger molecules, regulators, and structural components (including cytoskeletal proteins and cell wall biosynthetic enzymes). The pathway can also be activated via internal cues linked with other signaling pathways.

logues depends on their ability to enter the fungal cell. Differences in drug metabolism and specific CHS structure can also influence the degree of inhibition efficacy (Munro and Gow, 2001). As some plant pathogenic fungi are known to be sensitive to polyoxins, these compounds have been used (though to a limited degree) in agricultural practice (Cohen, 1987; Worthington, 1988). Overall, even though both polyoxins and nikkomycins can be effective CHS inhibitors in vitro, their limited efficacy in vivo (presumably on the basis of uptake and delivery limitations) has resulted in their restricted use as antifungals. Nonetheless, nikkomycins were effective in the treatment of murine histoplasmosis, and the combined use of nikkomycins with other antifungals (Li and Rinaldi, 1999) provides additional options for harnessing CHS inhibition as part of antifungal drug therapy programs.

GS Inhibitors

Two classes of glucan synthase (GS) inhibitors, the glycolipid papulacandins and the lipopeptide echinocandins, have been developed. They are noncompetitive inhibitors of GS, encoded by the essential *FKS1* gene (Beauvais and Latgé, 2001). Their precise mode of action is not well understood because of the difficulty in isolating active GS subunits. Interestingly, the echinocandins highlight differences between the cell walls of yeasts and filamentous fungi. They lyse and kill yeast cells but are only fungistatic to aspergilli, causing lysis of the hyphal tips and the formation of compact ball-like colonies (Bowman et al., 2002). Synthetic analogues of the echinocandins (but not of papulacandins) have been produced by several drug companies (caspofungin/Cancidas™ [Merck], FK 463/Micafungin™ [Fujisawa], and LY303366/Anidulafungin™ [Lilly]) and are used to treat invasive aspergillosis and disseminated candidiasis. Their clinical efficacy is similar to that seen for existing front-line antifungals from the polyene and azole families (Denning, 2003). The possibility of combining echinocandins with other antifungals is being actively investigated in animals (Ostrosky-Zeichner, 2008).

Mannosylation Inhibitors

Pradimicins are antifungal compounds that damage the cell wall of yeast and filamentous fungi by binding to terminal mannose residues, resulting in cell wall and membrane deformation and apoptosis (Walsh and Giri, 1997). Interestingly, in yeast, deletion of the mannosylated cell wall stress sensor *SLG1* increased resistance to pradimicin, suggesting that these compounds have a specific cell wall target (Hiramoto et al., 2005). The pradimicins were effective in curing fungal infections in animal studies, but their development for use in humans was discontinued because of liver toxicity (Walsh and Giri, 1997).

Cell Wall-Specific Antibodies

Antibodies have been developed both for identifying and diagnosing mold infections and for antifungal therapy (Nimrichter et al., 2005). For example, the Platelia enzyme-linked immunosorbent assay uses a monoclonal antibody that recognizes the galactofuranose side chains of *Aspergillus* galactomannan and galactomannoproteins released from the cell wall during infection. Monitoring of high-risk patients with Platelia can identify *A. fumigatus* infection before the onset of clinical symptoms, leading to early, more effective antifungal treatment (Mennink-Kersten et al., 2004).

A human recombinant antibody (Mycograb) recognizing the cell wall-associated heat shock protein Hsp90 of *C. albicans* has been produced and tested for antifungal activity and therapy. It is active against several yeast species and acts synergistically with amphotericin B and other antifungal drugs (Matthews et al., 2003). Mycograb is currently being used in clinical trials (Pachl et al., 2006), representing an excellent example of the use of cell wall ligands as new therapeutic agents against fungal infections.

Identification of Novel Cell Wall Targets

In principle, any essential, conserved, and well-characterized CWP involved in cell wall construction or host-pathogen interaction can serve as the target for the development of novel antifungals. For example, the *GAS/PHR/GEL* β-1,3 transglucosylases, which generate new branches of β-1,3 glucan, are promising drug target candidates: transglucosylase gene deletion in both yeast and filamentous fungi results in stunted growth, reduced virulence, and increased antifungal susceptibility (Beauvais and Latgé, 2001; Mouyna et al., 2005). Similarly, β-1,3 glucanases and chitinases, which degrade glucan and chitin, respectively, and enable plasticization and cell wall expansion, may also be potential drug targets. However, too little is known about their biochemistry and function to enable drug development.

SUMMARY, OPEN QUESTIONS, AND FUTURE RESEARCH

The broad spectrum of functions that the fungal cell wall performs is clearly reflected in its complexity. Though rich in seemingly inert constituents, the cell wall is an extremely active part of the fungal cell. Even though many cell wall components have been characterized to various extents, our understanding of cell wall biosynthesis is far from complete. The mechanism by which cell wall polysaccharides are extruded and the significance of the variations in cell wall constituents in different fungi are just examples of questions yet to be answered. Although regulatory networks that affect cell wall synthesis and maintenance have been identified, the comprehensive signaling machinery involved and the associations between different pathways have yet to be elucidated. The significance of the cell wall as a target for antifungals has long been appreciated, yet the number of successful drugs developed is very small. Lastly, important progress has been made in the analysis of the cell wall. However, our still-inadequate levels of understanding concerning the fundamental features of the cell wall, as well as our limited current capabilities in harnessing our knowledge for relevant applications in medicine, agriculture, and industry, provide us with many challenges for the future.

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18

Hyphal Growth and Polarity

STEVEN D. HARRIS

The filamentous fungi inhabit a diverse array of ecological niches. This capacity presumably reflects several intrinsic features of fungal biology, such as their metabolic versatility and their ability to tolerate environmental stress. Of these features, one of the most important is undoubtedly the ability to form highly polarized hyphae that enable filamentous fungi to rapidly and efficiently colonize new substrates. With the exception of flagellated zoospores formed by chytrids (Taylor et al., 2004), fungi do not generate cells that are motile. The formation of polarized hyphae thus seems to be an ingenious solution to the problems that would arise from an otherwise sessile lifestyle (for example, escape from local environments that have been depleted of nutrients). Accordingly, it is no surprise that polarized hyphal growth is the defining characteristic of filamentous fungi. It should be noted that hyphal growth is not an exclusive property of fungi; other organisms, including the bacterial actinomycetes and the alga-related oomycetes, also employ hyphae as a means of growth (for examples, see Chater and Chandra, 2006; and Gow, 2004). Even higher plants and animals form highly polarized structures (e.g., plant pollen tubes and animal axons) that superficially resemble hyphae (Cheung and Wu, 2008; Arimura and Kaibuchi, 2007). Nevertheless, no group of organisms has resorted to hyphal growth to the same extent as fungi have. Although it is not possible to pinpoint the exact evolutionary origins of hyphal growth in fungi, it is clear that only a subset of species within the Chytridiomycota, which is currently recognized as the most ancestral fungal phylum, grow by forming hyphae (James et al., 2006). By contrast, all subsequent fungal phyla (i.e., Zygomycota, Ascomycota, and Basidiomycota) almost uniformly exhibit polarized hyphal growth (note that for the purpose of this argument, yeast growth is considered to be a modified form of hyphal growth). Because most of these fungi are terrestrial, whereas chytrids are primarily aquatic, it is tempting to speculate that polarized hyphal growth played an important role in allowing fungi to adapt to a terrestrial lifestyle.

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Hyphal growth encompasses several different morphogenetic processes. Foremost among these is the establishment and maintenance of a stable axis of polarized growth. As a result, cell surface expansion and cell wall deposition are confined to a discrete location that ultimately becomes the hyphal tip. The formation of additional polarity axes in subapical regions, the original spore, or in some cases even from a growing tip, generates branches or secondary hyphae, which ultimately results in a differentiated mycelium consisting of multiple hyphae (Fig. 1). The importance of polarized axis formation in enabling hyphal growth has been amply documented using genetic, physiological, molecular, and cell biological tools and is also supported by computational studies (summarized by Harris, 1997; Bartnicki-Garcia, 2002; Momany, 2002; Harris, 2006; and Steinberg, 2007). Another critical process underlying hyphal growth is septum formation. Septa are formed via a cytokinetic mechanism that resembles cell division in animal cells (summarized by Harris, 2001; and Walther and Wendland, 2003). However, instead of separating, hyphal cells remain attached and the division site is marked by a cross wall (i.e., the septum). Although septum formation is not required per se for the formation of polarized hyphae or for hyphal growth, mutant analysis suggests that septa are needed to form a differentiated mycelium (Harris et al., 1994). Recently, it has become apparent that hyphal anastomosis is also an integral feature of hyphal morphogenesis and colony development (summarized by Glass et al., 2004; see also the following chapters). Whereas hyphae at the advancing edge of a mycelial colony tend to avoid one another, those in interior regions appear to engage in chemotropic interactions and subsequently undergo hyphal fusions. In some cases, even germinating conidia are capable of anastomosis (Gabriela Roca et al., 2005). Like septum formation, hyphal anastomosis is not necessary for hyphal growth. However, by permitting the exchange of nutrients and presumably signals as well, this process clearly contributes to the formation of a fully functional mycelium.

Because of its importance to the lifestyle of filamentous fungi, there is considerable interest in characterizing the mechanisms underlying hyphal growth. In a practical sense, this likely reflects increased interest in understanding fungal interactions with humans and plants. For example,

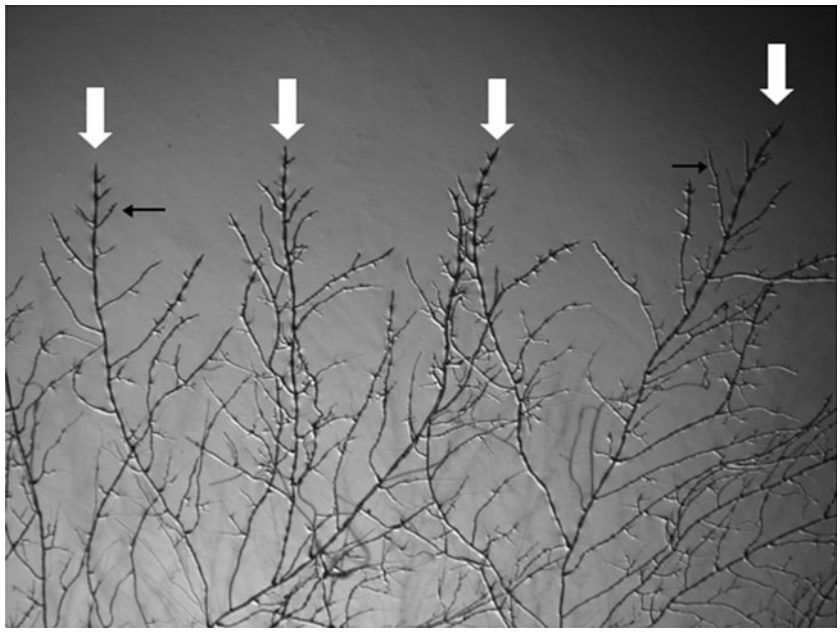

FIGURE 1 Organization of growing hyphae at the edge of a mycelial colony. Shown is the advancing edge of an *N. crassa* (strain FGSC9716) colony growing on Vogel's minimal medium supplemented with histidine. Thick white arrows indicate dominant extending hyphae. Thin black arrows show examples of secondary lateral branches. Note that branch emergence is suppressed in the immediate vicinity of hyphal tips.

while polarized hyphal growth is not generally recognized as a determinant of fungal virulence, it has been demonstrated that defects in hyphal morphogenesis do compromise the virulence of both human and plant pathogens (e.g., Nichols et al., 2004; Castillo-Lluva et al., 2007). Furthermore, recent studies suggest that the precise regulation of polarized hyphal growth appears to influence the outcome of fungal interactions with plants (Rittenour and Harris, 2008). As a result of the interest in understanding hyphal growth, a reasonably large number of proteins have been identified as playing key roles in the establishment and maintenance of hyphal polarity, septum formation, or hyphal anastomosis (summarized by Harris, 2006). Much of this effort has been guided by the so-called "yeast paradigm," which despite its limitations, has yielded valuable insight into hyphal morphogenesis (Harris and Momany, 2004). Although a coherent picture of the mechanisms underlying hyphal growth has yet to emerge, the rapid development of new tools and resources for manipulating filamentous fungi suggests that we will not have to wait much longer for this to happen. This chapter summarizes the progress achieved toward understanding the organization of fungal hyphae and the cellular systems involved in hyphal morphogenesis. Particular attention is paid to the mechanisms that have been implicated in the regulation of polarized growth and septum formation in filamentous fungi. Finally, the intriguing question of how morphogenetic regulatory systems may have evolved in the fungal kingdom is briefly addressed.

ORGANIZATION OF FUNGAL HYPHAE

Apical Dominance

As a general rule, fungal hyphae grow solely by tip extension (i.e., cell surface expansion and cell wall deposition are confined to the immediate hyphal apex). This mode of growth presumably reflects the importance of apical dominance, whereby the growing tip is dominant and appears to suppress the formation of other tips (i.e., lateral branches) in its general vicinity (Rayner, 1991). At a physiological level, apical dominance presumably reflects the exclusive targeting of cellular resources (i.e., vesicles laden with precursors required for cell surface expansion and cell wall deposition) to the hyphal tip at the expense of potential branching sites. Accordingly, branch sites would only become active once they are a sufficient distance from the growing tip. This would generate the stereotypical growth pattern of a fungal mycelium, with hyphae radiating outwards from the colony center and new hyphae arising via the formation of lateral branches (Fig. 1). It is not too difficult to imagine that the absence of apical dominance would lead to a disordered mass of hyphae growing in random directions. In fact, it might be instructive to consider apical dominance as performing the same role as the bud site selection system in the yeast *Saccharomyces cerevisiae*. Individual yeast cells exhibit one of two budding patterns depending on their mating type (Chant, 1999). Disruption of the mechanisms that specify these patterns do not perturb growth per se, but cells bud in a random fashion that alters colony formation and function (for an example, see Vopalenska et al., 2005). By analogy, apical dominance might represent a process that uses positional information to enable the directed growth of hyphae.

Despite its presumed importance, little is known about the mechanisms that may impose apical dominance on a hypha. In principle, any signaling molecule that exhibits a biased localization at growing hyphal tips could suppress branch formation and thereby promote apical dominance. Tip-high calcium and ion gradients are well known in filamentous fungi (Jackson and Heath, 1993; Robson et al., 1996), and it has been previously suggested that calcium could play a role in maintaining a single dominant hyphal

tip (Schmid and Harold, 1988). More recently, it has become apparent that reactive oxygen species (ROS), primarily superoxide, accumulate at hyphal tips and are seemingly involved in the enforcement of apical dominance (Semighini and Harris, 2008). Furthermore, studies involving several fungi, including *Aspergillus nidulans*, *Magnaporthe grisea*, and *Epichloe festucae*, implicate NADPH oxidase (Nox) and its associated regulators in the generation of ROS at hyphal tips (Tanaka et al., 2006; Egan et al., 2007; Takemoto et al., 2007; Semighini and Harris, 2008). An appealing, but still somewhat speculative model is that localized ROS generated by Nox triggers the formation of a calcium gradient at hyphal tips, as has been demonstrated in plant root hairs (Foreman et al., 2003). Indeed, as shown in *Arabidopsis thaliana*, ROS and calcium may act via feedback loops to mutually reinforce their respective gradients (Takeda et al., 2008). The net effect would be the generation of a positional signal that specifies the dominant axis of hyphal polarity.

Although apical dominance appears to be a characteristic feature of fungal hyphae, it has also become clear that it is not the only means of hyphal extension. Within the phylum Saccharomycotina (i.e., hemiascomycetes), both *Ashbya gossypii* and *Geotrichum candidum* form hyphae that undergo regular and repeated apical branching (split tips) (Heath et al., 2003; Philippsen et al., 2005). In these hyphae, apical dominance is presumably temporarily suspended to allow the simultaneous formation of two adjacent hyphal tips. Notably, the filamentous fungi within the Saccharomycotina do not possess obvious homologues of Nox and it is unclear whether they accumulate ROS at hyphal tips. Because they do possess apparent homologues of the yeast bud site selection markers (e.g., Bud8 [http://agd.vital it.ch/Ashbya_gossypii/geneview?db=core;gene=ACL193C]), it is tempting to speculate that they might be used to mark the tip and to regulate apical dominance in these fungi. An even more striking variation from the theme of apical hyphal extension is observed in the ryegrass mutualist *E. festucae*. Because this fungus must coordinate its growth with leaf cells in its grass host, it undergoes intercalary hyphal growth (Christensen et al., 2008). Specifically, ROS accumulation at hyphal tips appears to prevent the tip from extending, and growth is instead limited to internal hyphal segments demarcated by septa.

The Hyphal Tip Cell

Most filamentous fungi form multicellular hyphae that are compartmentalized by septa. Because it contains the extending hyphal tip, the apical compartment is known as the hyphal tip cell. This cell typically possesses a Spitzenkörper at the hyphal tip and displays asymmetric organization of vacuoles and endomembranes (i.e., endoplasmic reticulum [ER] and Golgi bodies) (Fig. 2). The Spitzenkörper, which was first described by Girhardt (1957) and has been extensively characterized by Bartnicki-Garcia and colleagues (Bartnicki-Garcia, 2002), is a dynamic structure of variable composition and shape. It is known to contain vesicles of different sizes, as well as microfilaments, microtubules, ribosomes, and possibly Woronin bodies (summarized by Harris et al., 2005). Although Spitzenkörper morphology can vary widely among different fungi, or even at different times in the same hyphal tip (Lopez-Franco and Bracker, 1996), it is almost always associated with hyphal tips undergoing active extension. Furthermore, mathematical models coupled with recent experimental observations strongly support the view that the Spitzenkörper determines the direction and rate of hyphal extension (Bartnicki-Garcia et al., 1995; Riquelme et al., 1998; Konzack et al., 2005). Nevertheless, a precise molecular description of what goes on in the Spitzenkörper has yet to emerge. At the very least, it appears to function as a vesicle supply center where exocytic vesicles transported from the hyphal interior on microtubules are transferred to microfilaments for localized distribution at the hyphal tip. In addition, results from microscopy studies suggest that the

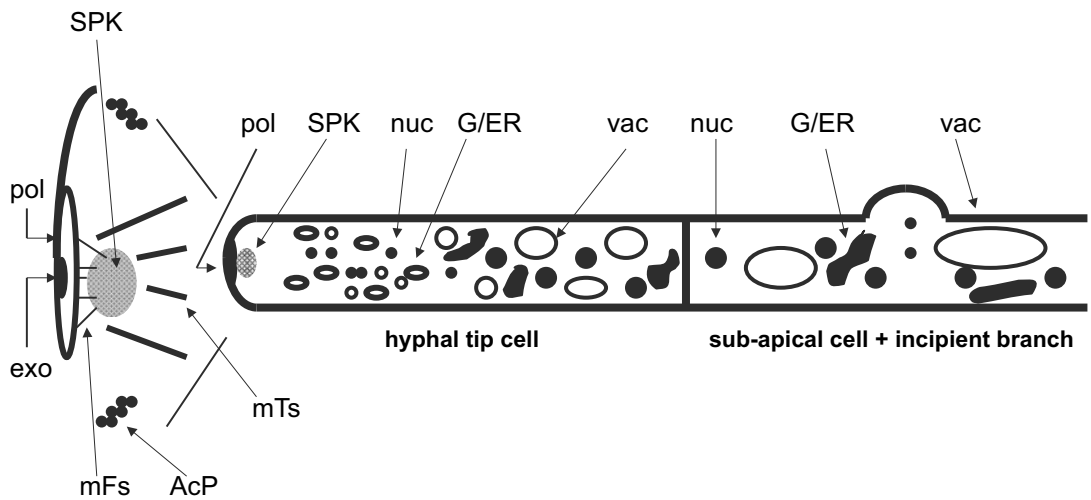

FIGURE 2 The organization of hyphal tip cells and subapical cells is shown through a schematic depiction of an extending hypha. Note the asymmetric organization of the hyphal tip cell, whereas the subapical cell remains uniformly organized until a new tip (i.e., the incipient branch) is formed. In hyphal tip cells, nuclei (nuc) exhibit a gradient of mitosis, with condensed mitotic nuclei located proximal to the tip. In addition, vacuoles (vac) and endomembranes (G/ER) are more fragmented near the tip. Finally, the tip also houses the Spitzenkörper (SPK) and the polarisome (pol). The enlarged depiction of the hyphal tip shows the exocyst (exo), microtubules (mTs), actin filaments (mFs), and actin patches (AcP). In the subapical cell, mitosis is blocked until a new branch emerges. At that time, nuclei proximal to the branch site resume mitosis. See the text for further details.

Spitzenkörper might also serve as a microfilament-organizing center (Harris et al., 2005).

The development of imaging probes that permit real-time localization studies has provided further insight into the organization of the hyphal tip cell. For example, the localization of chitin synthases in *Neurospora crassa* has revealed that endomembranous structures are organized in an asymmetric manner, with globular bodies in distal regions appearing to fragment into smaller vesicles as they approach the tip (Riquelme et al., 2007). The distribution of vacuoles is also asymmetric, as larger vacuoles appear to be primarily confined to the distal regions of tip cells. Finally, the presence of tip high calcium and ion gradients defines an additional asymmetry in tip cells (Jackson and Heath, 1993; Robson et al., 1996).

Nuclei in the hyphal tip cell are mitotically active and divide in a manner that appears to be coordinated with cellular growth. The term "duplication cycle" has been used to draw an analogy between the cell cycle of the hyphal tip cell and that of uninucleate yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* (Fiddy and Trinci, 1976; Harris, 1997). Although nuclear division is coupled to the doubling of cytoplasmic volume during the duplication cycle of hyphal tip cells, the mechanisms that link either of these events to polarized hyphal growth, if any, remain to be determined. This stands in sharp contrast to yeasts such as *S. cerevisiae*, where regulatory interactions between cyclin-dependent kinases (Cdks) and Rho GTPase modules enforce tight coordination of polarized growth with cell cycle progression (summarized by Yoshida and Pellman, 2008). A further complication in determining whether nuclear division is coupled to morphogenesis in hyphal tip cells is the observation of two seemingly distinct patterns of mitosis in these cells (summarized by Gladfelter, 2006a). In one pattern, as best exemplified in *A. nidulans*, the tip cell possesses a limited number of nuclei (typically 8 to 24) and mitosis proceeds in a parasynchronous wave starting from the tip (Clutterbuck, 1970). In the alternate pattern, as seen, for example, in *A. gossypii*, the tip cell is populated with many nuclei (often >50) and mitosis occurs in a completely asynchronous manner, such that individual nuclei divide regardless of the mitotic status of their neighbor (Gladfelter et al., 2006). At first glance, the former pattern would appear to be more conducive to the imposition of a global regulatory mechanism that coordinates morphogenesis with nuclear division, but this still remains to be tested.

Subapical Hyphal Cells

Subapical hyphal cells, also known as intercalary cells, are those cells in a hypha that are not located at the tip (i.e., they are bounded by septa at either end). In addition to their position, there are notable features that distinguish these cells from hyphal tip cells (Fig. 2). First, they possess no obvious asymmetry; there is no Spitzenkörper, and vacuoles and endomembranes appear to be uniformly distributed. Indeed, in many fungi, such as *Candida albicans* and *Ustilago maydis*, subapical cells contain little cytoplasm at all and are largely filled with vacuoles (Barelle et al., 2003; Steinberg and Fuchs, 2004). Second, nuclei present in subapical cells typically undergo cell cycle arrest. The nature of this arrest is not well understood, though it has been shown in *C. albicans* that it occurs in G_1 and is lifted when subapical cells attain a critical cytoplasmic volume (Barelle et al., 2003; Barelle et al., 2006). Perhaps the most important role of subapical hyphal cells is the generation of new hyphal tips, which typically happens as part of a normal morphogenetic program that produces lateral branches from a primary hypha. The mechanisms that underlie the formation of a new hyphal tip from subapical cells remain poorly understood. However, several important steps are likely to be involved (Seiler and Plamann, 2003). For example, the site of branch emergence must be specified. In some fungi (i.e., *A. gossypii*), lateral branches emerge adjacent to preexisting septa, suggesting that septation itself may provide a marker (Wendland, 2003), whereas branch sites appear to be stochastically determined in other fungi (i.e., *A. nidulans* [Trinci, 1978; Walther and Wendland, 2003]). In addition, a new Spitzenkörper must be generated and the morphogenetic machinery must be targeted to the branch site. Localized calcium fluxes have been implicated in this step, though their precise role has yet to be determined (Grinberg and Heath, 1997; Virag and Griffiths, 2004). Finally, the cell cycle arrest that is characteristic of subapical cells must be lifted to allow resumption of nuclear division. In *C. albicans*, this appears to be triggered by passage though a G_1 cell size control (Barelle et al., 2006). The relevance of this observation to other filamentous fungi is a key issue that requires further study.

THE MORPHOGENETIC MACHINERY

Actin Cytoskeleton

The organization of the actin cytoskeleton has been extensively studied in numerous filamentous fungi. Classical studies reliant upon immunofluorescence microscopy and recent studies using green fluorescent protein (GFP)-based probes have both revealed a similar pattern: actin filaments found at the immediate hyphal tip (and occasionally in association with the Spitzenkörper) are subtended by a belt of actin patches (for examples, see Harris et al., 1994; Bruno et al., 1996a; Torralba et al., 1998; Wendland and Philippsen, 2001; Cheng et al., 2001; Pearson et al., 2004; Harris et al., 2005; Taheri-Tahesh et al., 2008; and Upadhyay and Shaw, 2008). Mobile actin patches are also observed in distal regions of the hyphal tip cell (Taheri-Tahesh et al., 2008; Upadhyay and Shaw, 2008). An additional actin-based structure, the contractile actin ring, transiently appears in hyphal tip cells during septum formation (Harris, 2001). Results from genetic, molecular, and chemical analyses emphasize the importance of the actin cytoskeleton to hyphal morphogenesis (Torralba et al., 1998; Virag and Griffiths, 2004; Araujo-Bazan et al., 2008). The complete absence of actin filaments does not abolish growth per se but does block polarized hyphal growth and also prevents septum formation (Harris et al., 1994; Torralba et al., 1998). In particular, emerging evidence strongly implicates actin patches as sites of endocytosis and suggests that by analogy to yeast, a "comet tail" of highly branched actin filaments might drive vesicle internalization (Upadhyay and Shaw, 2008; Araujo-Bazan et al., 2008). Although direct evidence is still lacking, it seems likely that longer unbranched actin filaments (i.e., actin cables) mediate the localized transport of exocytic vesicles at the immediate hyphal tip (A. Virag and S. D. Harris, unpublished results). Finally, the essential function of the contractile actin ring during septum formation remains unknown. However, the analogous structure in both *S. pombe* and *S. cerevisiae* is known to provide a spatial landmark that recruits the machinery needed for deposition of the septal cell wall (Vallen et al., 2000; Vjestica et al., 2008).

Two general types of actin filament nucleating proteins are widely conserved in eukaryotes: the formins and Wiscott-Aldrich Syndrome protein (WASp). In general, formins nucleate actin cables and contractile actin rings, whereas

WASp directs the formation of branched actin filaments (summarized by Pollard, 2007; and Chhabra and Higgs, 2007). The underlying biochemical mechanisms have been extensively described in both yeasts and animals (Moseley and Goode, 2006; Pollard, 2007). Among filamentous fungi, formins have been characterized in *C. albicans*, *A. gossypii*, and *A. nidulans* (Harris et al., 1997; Sharpless and Harris, 2002; Li et al., 2005; Martin et al., 2005; Schmitz et al., 2006). *C. albicans* possesses two formins that apparently exhibit distinct localization patterns; Bni1 localizes to hyphal tips, and Bnr1 localizes to septa (Li et al., 2005; Martin et al., 2005). Nevertheless, neither is required for polarized hyphal growth or for the formation of septa in hyphae (the double *bni1 bnr1* mutant phenotype remains unknown). By contrast, despite the existence of two apparent homologues, *A. gossypii* Bni1 is required for the establishment of hyphal polarity (Schmitz et al., 2006). It localizes to hyphal tips, where it nucleates actin cables that appear to mediate localized vesicle delivery. Unlike *C. albicans* or *A. gossypii*, *A. nidulans* possesses only a single formin, SepA (note that all sequenced Pezizomycotina genomes possess only a single annotated formin [S. D. Harris, unpublished result]). Notably, *sepA* mutants can form hyphae, though they exhibit defects in the maintenance of hyphal polarity and cannot undergo septation because of failure to form the contractile actin ring at septation sites (Harris et al., 1997; Sharpless and Harris, 2002). An important implication of this phenotype is that polarized hyphal growth can occur in the absence of any formin-mediated actin nucleating activity. Because chemical inhibitor studies show that actin filaments are required for polarized hyphal growth (for an example, see Torralba et al., 1998), WASp-mediated nucleation of branched actin filaments presumably enables polarity establishment to occur in *sepA* mutants. Although WASp homologues have not yet been subjected to functional studies in members of the Pezizomycotina, analysis of the *C. albicans* homologue, Wal1, supports the existence of distinct formin- and WASp-mediated pools of actin filaments (Walther and Wendland, 2004; Martin et al., 2005).

Several different types of actin-associated proteins are known to modulate the stability and dynamics of actin filaments (for examples, see Kreis and Vale, 1995; and Moseley and Goode, 2006). These include filament bundling proteins, such as fimbrin and alpha-actinin, as well as proteins that disassemble or sever filaments, such as ADF/cofilins or gelsolin. Annotation of the *A. nidulans* and *N. crassa* genome sequences has revealed that filamentous fungi possess a larger complement of these proteins than do yeasts (Borkovich et al., 2004; Harris et al., 2009), which further underscores the greater complexity of actin filament dynamics in hyphae. In *A. nidulans*, recent results highlight the important role of fimbrin (FimA) in the establishment of hyphal polarity (Upadhyay and Shaw, 2008). In particular, FimA localizes to actin patches, where it mediates endocytosis by presumably bundling branched actin filaments. Notably, alpha-actinin (AcnA in *A. nidulans*) has a role in polarized hyphal growth and septum formation that is independent from that of fimbrin (A. Virag and S. D. Harris, unpublished results). These observations seemingly imply the existence of distinct pools of actin filaments that are bundled in a different manner, with one pool (regulated by FimA) supporting endocytosis and the other (regulated by AcnA) directing exocytosis.

Filamentous fungi possess a large repertoire of myosins that include members of multiple classes, including a novel class (class VI) composed of hybrid myosin/chitin synthases that are involved in polarized hyphal growth and septum formation (see chapter 16 for further details). The class I myosins (i.e., MyoA in *A. nidulans* and Myo5 in *C. albicans*) are required for the establishment of hyphal polarity and localize to hyphal tips (McGoldrick et al., 1995; Oberholzer et al., 2002), where MyoA might serve as a scaffold for WASp-dependent actin filament nucleation (Liu et al., 2001). Class V myosins (e.g., Myo5 in *U. maydis* and Myo2 in *C. albicans*) have also been implicated in polarized hyphal growth and localize to hyphal tips (Weber et al., 2003; Woo et al., 2003), where it has been suggested that they might mediate vesicle transport between the Spitzenkörper and the cell surface.

Microtubule Cytoskeleton

In fungal hyphae, cytoplasmic microtubules typically exist as longitudinal arrays oriented parallel to the axis of hyphal extension (Grove and Bracker, 1970; Howard, 1981; Hoch and Staples, 1983). It is generally thought that these microtubules originate from microtubule-organizing centers associated with nearby nuclei (summarized by Xiang and Fischer, 2004). Localization of microtubule plus-end binding proteins in *A. nidulans* and *U. maydis* shows that these microtubules extend into the hyphal tip and appear to make cortical contacts (Han et al., 2001; Zhang et al., 2003; Schuchardt et al., 2005). Nevertheless, recent evidence suggests that the tip region also harbors microtubule nucleation sites (Straube et al., 2003; Konzack et al., 2005), though the relationship between these sites and the Spitzenkörper remains unclear at this time. Upon entry into mitosis, cytoplasmic microtubules are depolymerized so that tubulin can be shuttled into nuclei to enable construction of the mitotic spindle (Riquelme et al., 2003; Ovechkina et al., 2003; Sampson and Heath, 2005). However, at least in *A. nidulans*, intact cytoplasmic microtubules are maintained at the hyphal tip, where they allow tip extension to continue as mitosis proceeds (Horio and Oakley, 2005). One possibility is that the tip-associated nucleation sites organize this particular pool of microtubules.

Two specific roles have been attributed to cytoplasmic microtubules in support of polarized hyphal growth. First, it is generally accepted that microtubules provide "tracks" for the long-range transport of vesicles from the interior regions of hyphae to the tip (summarized by Steinberg, 2007). Second, microtubules have been strongly implicated in positioning the Spitzenkörper within the hyphal tip, such that in their absence, hyphae cannot extend in a directed manner and exhibit irregular growth patterns (Riquelme et al., 1998; Konzack et al., 2005; Takeshita et al., 2008). Indeed, the latter phenotype (i.e., bent, curved, or kinked hyphae) is typically observed when microtubules are eliminated by mutations or chemical perturbations. However, emerging evidence highlights a far more fundamental role for microtubules in polarized hyphal growth. For example, the ability to sustain rapid hyphal tip extension rates in *A. nidulans* is dependent upon the presence of intact cytoplasmic microtubules (Horio and Oakley, 2005). In their absence, hyphae extend at a slower rate, characteristic of germlings. In addition, it has also been demonstrated that microtubules become essential for the establishment of hyphal polarity under conditions that compromise actin filament formation (i.e., *sepA* mutations in *A. nidulans* [Virag et al., 2007]).

Filamentous fungi possess an extensive set of microtubule-based motor proteins (see chapter 16 for further details). Plus-end-directed kinesins are largely responsible for anterograde transport towards the hyphal tip, whereas minus-end-directed dyneins mediate retrograde transport from the tip (summarized by Steinberg, 2007). In *U. maydis*,

the complete set of 10 kinesins have been characterized (Schuchardt et al., 2005), and as many as 7 kinesins may be involved in polarized hyphal growth. Results from this study suggest the existence of considerable redundancy among kinesins and dyneins and also emphasized the point that myosins and kinesins may act in parallel to mediate vesicle transport to the hyphal tip. In *A. nidulans*, the kinesin KipA is required for the localization, but not the transport, of the cell end markers TeaA and TeaR at the hyphal tip (Takeshita et al., 2008). These markers are part of a hyphal tip-anchoring complex that associates with sterol-rich membrane domains and maintains the position of the Spitzenkörper. Thus, in addition to vesicle transport, kinesins also mediate the ability of microtubules to ensure that hyphae extend in a directed manner.

Vesicle Trafficking

Polarized hyphal growth is driven by localized cell surface expansion and cell wall deposition at the hyphal tip. The precursors needed to support these processes are delivered to the tip in vesicles via exocytosis. The volume of vesicles undergoing transport to the tip is potentially staggering, given that hyphae are capable of extension rates as high as 7.5 mm/h (Lopez-Franco et al., 1994). Simultaneously, excess membrane material, along with other factors, such as possible landmark proteins, are presumably retrieved from the hyphal tip and recycled via endocytosis. Several recent studies have clarified the specific roles of exocytosis and endocytosis in polarized hyphal growth, and they have also revealed where these events occur in relation to the hyphal tip (Taheri-Tahesh et al., 2008; Upadhyay and Shaw, 2008; Araujo-Bazan et al., 2008).

The vesicle supply center model of hyphal tip extension in based on the premise that the radial distribution of vesicles from the Spitzenkörper is sufficient to generate the characteristic shape of the hyphal tip (Bartnicki-Garcia, 2002). According to this model, a gradient of exocytic vesicles would be delivered to the hyphal tip, with the peak immediately anterior to the Spitzenkörper. However, the actual site of vesicle delivery may be far more discrete than this model implies. In *S. cerevisiae*, Sec3 is a well-characterized exocyst component that provides a positional landmark for polarized exocytosis (Finger et al., 1998). The *A. nidulans* homologue of Sec3, SecC, localizes to a small region at the apex of the hyphal tip (Taheri-Talesh et al., 2008). Assuming that SecC shares a landmark function in common with yeast Sec3, this result considerably narrows the zone in which exocytic vesicles would presumably fuse with the plasma membrane. Furthermore, it has become increasingly apparent that exocytosis is spatially coupled to endocytosis at hyphal tips in a manner that resembles neuromuscular junctions and mating yeast cells (Gundelfinger et al., 2003; Valdez-Taubus and Pelham, 2003). Studies with *A. nidulans* reveal that several established markers for endocytosis, including FimA, AbpA (homologue of *S. cerevisiae* Abp1), AmpA (= *S. cerevisiae* Rvs167), and SlaB (= *S. cerevisiae* Sla2), colocalize with actin patches in a collar immediately behind the hyphal apex (Upadhyay and Shaw, 2008; Araujo-Bazan et al., 2008). Notably, vesicles that fuse with the plasma membrane at the site marked by SecC appear to be recycled by endocytosis at this collar (Taheri-Talesh et al., 2008). Other *A. nidulans* proteins potentially involved in specification of the endocytic zone include MesA and the septins (Westfall and Momany, 2002; Pearson et al., 2004). The presence of an endocytic zone immediately posterior to the exocytic zone provides an efficient mechanism for the retrieval of membrane material and cell surface proteins so that they can be recycled. A growing body of evidence supports the notion that a tip growth apparatus that encompasses the Spitzenkörper and other components, such as the polarisome, functions to maintain the coupling of endocytosis to exocytosis in rapidly growing hyphae (Araujo-Bazan et al., 2008; Taheri-Talesh et al., 2008). Because the tip growth apparatus is not observed in germlings, at least in *A. nidulans*, its formation apparently coincides with the transition to rapid hyphal extension.

The importance of exocytosis in supporting rapid hyphal tip extension is widely accepted. The dynamic kinesin-mediated transport of vesicles into the hyphal tip has been observed in *N. crassa* (Seiler et al., 1999). Furthermore, genetic screens for *A. nidulans* or *N. crassa* mutants defective in hyphal morphogenesis have consistently identified functions implicated in the formation and transport of exocytic vesicles. For example, in *A. nidulans*, mutations that compromise coatomer function or disrupt organization of the Golgi body prevent the formation of a stable polarity axis (Whittaker et al., 1999; Shi et al., 2004; Yang et al., 2008). In addition, a comprehensive *N. crassa* screen identified several functions required for exocytosis (i.e., coatomers and exocyst components) as being essential for polarized hyphal growth (Seiler and Plamann, 2003). On the other hand, support for the importance of endocytosis in rapid hyphal tip extension has only recently emerged. A significant advance was the demonstration that the lipophilic dye FM4-64, which monitors bulk membrane internalization, is recycled to the hyphal tip, where it labels the Spitzenkörper (Fischer-Parton et al., 2000). This observation suggested that endocytosis is involved in organizing the hyphal tip. Genetic analyses of *U. maydis* and *A. nidulans* support this notion. An *U. maydis* mutant with a defective endosomal t-SNARE exhibits morphological phenotypes that are suggestive of a failure to maintain hyphal polarity (Wedlich-Soldner et al., 2000; Fuchs et al., 2006). In *A. nidulans*, mutations affecting SlaB or FimA cause dramatic defects in the establishment of hyphal polarity (Upadhyay and Shaw, 2008; Araujo-Bazan et al., 2008). As an aside, despite its apparent importance to polarized hyphal growth, mutations that affect endocytosis are vastly underrepresented in the large collections of morphogenetic mutants that have been generated in *N. crassa* and *A. nidulans* when compared to mutations that disrupt exocytosis. The basis for this discrepancy is unknown, but one possibility is that endocytosis is essential for hyphal growth and is not as robust a process as exocytosis.

The source of exocytic vesicles that are transported to the hyphal tip is generally presumed to be the Golgi body. However, the location and nature of the Golgi body in filamentous fungi remain somewhat confusing issues. *C. albicans* hyphae possess discrete ER and Golgi complexes that are physically separated; the ER is largely confined to the cell body, whereas Golgi complexes are exclusively found near the hyphal tip (Rida et al., 2006). By contrast, other filamentous fungi such as *N. crassa* and *A. nidulans* contain endomembranous compartments that may in fact be contiguous ER and Golgi bodies (summarized by Markham, 1995). Recent evidence obtained using GFP-labeled probes predicted to reside in these compartments (i.e., chitin synthases and sialyltransferase) further suggests that they are motile and fragment into vesicles as they approach the tip (Riquelme et al., 2007; Hubbard and Kaminskyj, 2008). This would be consistent with the cisternal maturation model of Golgi transport, whereby a single Golgi compartment undergoes maturation from early to late as defined by its resident glycoprotein-modifying enzymes (the alternate "traditional" model posits the existence of distinct early,

middle, and late compartments [summarized by Malhotra and Mayor, 2006]). This would also imply that exocytic vesicles might originate from sites that are far more proximal to the hyphal tip than previously thought. A final observation that further underscores the complexity of vesicle dynamics in filamentous fungi is the likely existence of different Golgi complexes. Distinct populations of exocytic vesicles are found in the Spitzenkörper (summarized by Harris et al., 2005): a class of larger vesicles (70 to 90 nm) known as apical vesicles, which presumably mediate bulk secretion, and a class of smaller vesicles (30 to 40 nm) known as chitosomes, which appear to possess chitin synthase activity. This distinction appears to exist at the level of Golgi complexes as well; GFP-labeled chitin synthases localize to Golgi complexes different from those labeled with conventional Golgi markers (Riquelme et al., 2007).

REGULATION OF POLARIZED GROWTH

Ras- and Rho-Related GTPases

Monomeric GTPases possess a universally important role in the regulation of growth and cellular morphogenesis in eukaryotes (Ridley, 2001; Hall, 2005), including fungi. Extensive analyses of *S. cerevisiae* and *S. pombe* have implicated both Ras- and Rho-related GTPases in the control of cell proliferation and morphogenesis (Chang and Philips, 2006; Garcia et al., 2006; Park and Bi, 2007). In both yeasts, the Rho-related GTPase Cdc42 sits at the nexus of the signaling pathways that regulate polarized growth. It is locally activated by cell-type-specific positional landmarks and in turn activates several effectors that recruit the morphogenetic machinery to enable cell surface expansion and cell wall deposition. In addition, as originally observed in animal cells (summarized by Chant and Stowers, 1995), yeast GTPases are apparently linked in a regulatory cascade (i.e., Ras → Cdc42 → Rho) that drives polarized growth (Chant, 1999; Pruyne and Bretscher, 2000). The characterization of Ras- and Rho-related GTPases in filamentous fungi is still in its infancy. Nevertheless, important differences from the model yeasts have already emerged.

The first Ras homologue characterized in filamentous fungi was RasA from *A. nidulans* (Som and Kolaparthi, 1994). Genetic analyses suggest that RasA acts in parallel with protein kinase A to regulate the germination of conidiospores and also has additional roles in the regulation of development (Fillinger et al., 2002). Notably, constitutively activated RasA (i.e., RasA-GTP) triggers prolonged isotropic expansion of the spore and inhibits the establishment of hyphal polarity (Som and Kolaparthi, 1994; Osherov and May, 2000). The basis of this phenotype still remains unknown, but it likely reflects an inability to spatially and temporally coordinate growth signals with morphogenesis. Subsequent to the identification of RasA, Ras homologues have been characterized in several filamentous fungi (for examples, see Alspaugh et al., 2000; Lee et al., 2002; Ha et al., 2003; Park et al., 2006; and Bluhm et al., 2007), and like RasA, most of these are homologues of *S. cerevisiae* Ras1 or Ras2 that function to coordinate morphogenesis with growth and/or development (often as upstream regulators of mitogen-activated protein [MAP] kinase signaling modules). For example, Ras function is required in several dimorphic fungi to enable the switch from yeast-like growth to filamentous hyphal growth (Alspaugh et al., 2000; Lee and Kronstad, 2002; Muller et al., 2003).

Filamentous fungi possess an additional Ras homologue that appears to have a more intimate role in morphogenesis than RasA. These proteins share homology with *S. cerevisiae* Rsr1, which couples bud site selection to polarity establishment in yeast (Park and Bi, 2007). *A. gossypii* Rsr1 (AgRsr1) is required for the maintenance of a stable polarity axis (Bauer et al., 2004). A*grsr1* mutants exhibit slow colony growth and produce misshapen hyphae that are often kinked or bent. This morphology results from the frequent pausing of hyphal extension followed by reinitiation along a different axis from the one that was previously used. AgRsr1 localizes to the hyphal tip, where it appears to organize actin filaments by stabilizing the polarisome. *C. albicans* Rsr1 (CaRsr1) has also been implicated in the regulation of hyphal guidance (Hausauer et al., 2005). In addition, CaRsr1 mediates the ability of *C. albicans* hyphae to reorient their growth direction in response to external environmental cues such as electrical fields (galvanotropic growth) or surface contours (thigmotropic growth) (Brand et al., 2008). The only other predicted Rsr1 homologue to be characterized is *A. fumigatus* RasB. Remarkably, *rasB* mutants cannot maintain a stable axis of hyphal polarity and undergo frequent branching from the hyphal tip (Fortwendel et al., 2005). Whether this reflects a role in stabilization of the polarisome remains to be determined. Nevertheless, at least in those filamentous fungi that belong to the Saccharomycotina and Pezizomycotina, Rsr1 seemingly promotes directed hyphal extension as part of a spatial regulatory mechanism that responds to internal and external cues.

In animal cells, Cdc42 and the closely related GTPase Rac1 act in a sequential manner to generate cell polarity (Chant and Stowers, 1995). Homologues of Cdc42 have been identified and characterized in a diverse array of filamentous fungi (for examples, see Boyce et al., 2001; Scheffer et al., 2005a; Chen et al., 2004; and Virag et al., 2007). Moreover, unlike the yeasts *S. cerevisiae* and *S. pombe*, filamentous fungi also possess a Rac1 homologue (Boyce et al., 2003; Chen and Dickman, 2004; Mahlert et al., 2006; Virag et al., 2007; Rolke and Tudzynski, 2008; Tanaka et al., 2008). The relative importance of Cdc42 and Rac1 in the regulation of polarized hyphal growth appears to vary between different fungi. Within the Saccharomycotina, Cdc42 clearly has the predominant role in promoting hyphal polarity (Park and Bi, 2007). Neither *A. gossypii* nor *C. albicans cdc42* mutants are capable of generating polarized hyphae (Wendland and Philippsen, 2001; Ushinsky et al., 2002); the *A. gossypii* mutant forms swollen spores with a disorganized actin cytoskeleton. The localization of *C. albicans* Cdc42 to the polarisome (it may also associate with the Spitzenkörper) is consistent with the notion that it acts via multiple effectors to locally organize the morphogenetic machinery (Crampin et al., 2005). By contrast, the *C. albicans* Rac1 homologue is completely dispensable for hyphal growth except under conditions where cells are embedded in a matrix (Bassilana and Arkowitz, 2006). In addition, *C. albicans* Rac1 and Cdc42 share no functional overlap. Thus, Saccharomycotina Rac1 homologues appear to affect hyphal growth only under specific circumstances.

Outside the Saccharomycotina (i.e., in the Pezizomycotina or the Basidiomycetes), both Rac1 and Cdc42 are involved in polarized hyphal growth, though their relative contributions vary quite considerably. For example, in *U. maydis*, Rac1 is necessary and sufficient for the formation of dikaryotic hyphae, whereas Cdc42 is only needed for cell separation following septum formation (Mahlert et al., 2006). However, because a *rac1 cdc42* double-deletion mutant is only capable of isotropic growth, *U. maydis* Rac1 and Cdc42 share at least one function required for the establishment of hyphal polarity. In *E. festucae* and the related grass

pathogen *Claviceps purpurea*, deletion of *rac1* severely compromises hyphal extension and triggers massive hyperbranching (Rolke and Tudzynski, 2008; Tanaka et al., 2008). By contrast, other than a modest hyperbranching phenotype, deletion of *C. purpurea cdc42* does not dramatically alter hyphal morphology (Scheffer et al., 2005a). Unlike these fungi, Cdc42 does have a prominent morphogenetic function in *A. nidulans*, where it is required for normal hyphal morphology and for the formation of lateral branches (Virag et al., 2007). Furthermore, *A. nidulans* Cdc42 localizes to a surface crescent at the hyphal tip, and genetic analyses suggest that it likely acts upstream of the polarisome. Although *A. nidulans* Rac1 has no apparent role in hyphal morphogenesis under normal conditions, it can function as a substitute of Cdc42 and, as observed in *U. maydis*, a *cdc42 rac1* double-deletion mutant is inviable (Virag et al., 2007). In the dimorphic human pathogen *Penicillium marneffei*, Rac1 localizes to septation sites and is involved in septum formation (Boyce et al., 2003). In addition, it also localizes to hyphal tips, where it acts in coordination with Cdc42 to maintain normal hyphal morphology (Boyce et al., 2005a). Collectively, these observations suggest that there is no general consensus regarding the relative roles of Cdc42 and Rac1 in polarized hyphal growth. Presumably, at least one of these GTPases must localize to the hyphal tip to organize the actin cytoskeleton and the vesicle trafficking machinery. Thereafter, their precise functions in morphogenesis and development have probably evolved in response to specific ecological challenges faced by different filamentous fungi.

Filamentous fungi typically possess additional Rho GTPases besides Cdc42 and Rac1 (i.e., Rho1-4) (for an example, see Borkovich et al., 2004). However, the function of these GTPases is generally not that well characterized. As initially established in yeast, Rho1 homologues likely contribute to hyphal morphogenesis as regulatory subunits of β-(1-3)-glucan synthase (for an example, see Qadota et al., 1996). Biochemical studies with *A. fumigatus* and *C. albicans* demonstrate that Rho1 copurifies with the glucan synthase complex (Kondoh et al., 1997; Beauvais et al., 2001), and genetic analyses show that *rho1* mutations compromise cell wall integrity (Guest et al., 2004). Rho1 impacts additional morphogenetic pathways in *S. cerevisiae* (i.e., activation of the formin Bni1 and regulation of exocyst function [Park and Bi, 2007]); thus, it is likely that the aberrant hyphal morphologies observed in fungal *rho1* mutants are not solely due to defective regulation of glucan synthases. Among the other Rho GTPases found in filamentous fungi, initial insight into the function of Rho3 and Rho4 has been obtained. In *A. gossypii*, Rho3 acts to maintain polarisome stability at hyphal tips as a downstream effector of the signaling protein Boi1/2 (Knechtle et al., 2006). In the absence of Rho3, hyphal polarity is transiently lost. However, unlike the *rsr1* mutant described above, *rho3* mutants reestablish hyphal polarity along the previously existing axis, thereby suggesting that positional cues are not lost. *C. albicans* Rho3 is also required for normal hyphal morphogenesis (Dunkler and Wendland, 2007). Rho4 is a unique Rho GTPase that is not conserved in *S. cerevisiae* (yeast Rho4 is a paralogue of Rho3). Characterization of *N. crassa* Rho4 shows that it is absolutely essential for the formation of the contractile actin ring at septation sites (Rasmussen and Glass, 2005). In addition, Rho4 contributes to hyphal morphogenesis by regulating microtubule organization and stability (Rasmussen et al., 2008). Preliminary observations suggest that Rho4 performs similar functions in *A. nidulans* (H. Si and S. D. Harris, unpublished results).

In *S. cerevisiae* and *S. pombe*, Ras and Rho-related GTPases function in a coordinated manner to direct cellular morphogenesis. For example, Ras acts upstream of Cdc42 in both yeasts (for examples, see Chang et al., 1994; and Mosch et al., 1996), and antagonistic interactions between Cdc42 and Rho1 have been described in *S. cerevisiae* (Gao et al., 2004). Although similar relationships undoubtedly exist in filamentous fungi, only a limited number of examples have so far been demonstrated. These include the observation that the effects of Ras1 on hyphal morphology and branch formation are mediated in part by Cdc42 in *P. marneffei* (Boyce et al., 2005a). Moreover, in the plant pathogen *Colletotrichum trifolii*, Ras1 regulates hyphal morphology by activating Rac1, which in turn controls MAP kinase activation and ROS production (Chen and Dickman, 2004). More extensive analysis is clearly needed to investigate the functional relationships between these GTPases in filamentous fungi. Furthermore, it would not be surprising to find that these relationships vary between different fungi as a function of their lifestyle and evolutionary history.

GEFs and GAPs

The activity of monomeric GTPases is controlled by a set of proteins that determine their GTP-binding status and regulate their association with membranes (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007). Guanine nucleotide exchange factors (GEFs) are positive regulators that activate monomeric GTPases by exchanging GDP for GTP. GTPase-activating proteins (GAPs) are negative regulators that boost the intrinsic GTP hydrolysis rate of GTPases and thus facilitate conversion back to a GDP-bound state. By virtue of their ability to determine where and when Ras and Rho-related GTPases are active, GEFs and GAPs play a key role in the coordination of morphogenesis with growth and development. For example, several studies with *S. cerevisiae* demonstrate that Cdc42 GEFs (e.g., Cdc24) and GAPs (e.g., Rga1, Rga2, and Bem3) are dually regulated by Cdks to coordinate bud emergence with cell cycle progression (McCusker et al., 2007; Sopko et al., 2007; Knaus et al., 2007). In addition to GEFs and GAPs, Rho dissociation inhibitors also down-regulate Rho-related GTPases by extracting them from membranes while they are in a GDP-bound state, such that they can no longer be accessed by GEFs for reactivation (Dovas and Couchman, 2005).

The deepest insight into the roles of GEFs and GAPs in polarized hyphal growth comes from studies of Cdc42 regulation in *C. albicans*. As might be expected for a positive regulator of Cdc42, *C. albicans* strains with reduced levels of Cdc24 have defects in polarized hyphal growth similar to those of *cdc42* mutants, and like Cdc42, Cdc24 also localizes to hyphal tips (Bassilana et al., 2005). By contrast, the Cdc42 GAP Rga2 appears to be excluded from hyphal tips (Zheng et al., 2007). Notably, the Hgc1-Cdc28 Cdk complex associates with and inactivates Rga2 to enable localized activation of Cdc42 and germ tube emergence. These observations show that hyphal growth in *C. albicans* is dependent upon the concerted regulation of both Cdc42 GEFs and GAPs. Cdc24 mutants have also been characterized in *A. gossypii* and *N. crassa*; in both cases the establishment of hyphal polarity is abolished, and at least in *A. gossypii*, the actin cytoskeleton remains depolarized (Wendland and Philippsen, 2001; Seiler and Plamann, 2003). Mutations affecting Cdc42 GAPs have not been generated in other filamentous fungi (note that the *A. gossypii* Rho1 GAP Bem2 has been shown to regulate the establishment and maintenance of hyphal polarity [Wendland and Philippsen, 2000]). However, given the prevailing view that negative

regulation of Cdc42 (or possibly Rac1) plays a critical role in breaking cellular symmetry, it seems reasonably likely that they will be intimately involved in hyphal morphogenesis.

The role of Ras GEFs and GAPs in polarized hyphal growth has also been addressed to a limited extent. In *U. maydis*, Sql2 is a likely GEF for Ras2, although its deletion does not adversely affect hyphal morphogenesis (Muller et al., 2003). However, two different Ras GAPs have been implicated in the regulation of polarized hyphal growth. *C. albicans* Bud2 specifies the use of normal branch sites (i.e., adjacent to septa) through its predicted ability to promote GTP cycling of Rsr1 (Hausauer et al., 2005). In *A. nidulans*, the Ras GAP GapA localizes to hyphal tips, where it presumably down-regulates RasA to permit normal actin organization and hyphal growth (Harispe et al., 2008). In addition, the loss of GapA allows germinating spores to initiate isotropic expansion in the absence of a growth signal. The analysis of *gapA* and *rasA* mutants in *A. nidulans* suggests that active Ras interferes with the formation of a stable axis of hyphal polarity. Accordingly, it seems likely that Ras GAPs will be key determinants that coordinate growth with polarized hyphal growth.

Protein Kinases

Several different classes of protein kinases have been implicated in the regulation of polarized hyphal growth in filamentous fungi. In many cases, particularly for dimorphic fungi such as *C. albicans*, *U. maydis*, and *C. neoformans*, MAP kinases have a vital role in the signaling pathways that coordinate morphogenesis with growth conditions under both normal and adverse conditions. Fungal MAP kinases have been extensively reviewed elsewhere (for an example, see Bahn et al., 2007) and are thus not considered here. However, two other classes of protein kinases that do have a more direct role in hyphal morphogenesis, the p21-activated kinases (PAKs) and the nuclear Dbf2-related kinases (NDRs), merit further attention. The PAKs are well-known effectors of Cdc42 and Rac GTPases (Arias-Romero and Chernoff, 2008), whereas NDRs are AGC family kinases that in some cases function downstream of PAKs (Hergovich et al., 2006).

Two PAKs, Ste20 and Cla4, both of which were initially characterized in *S. cerevisiae*, are involved in polarized hyphal growth. In *C. albicans*, Ste20 functions as a MAP kinase activator (i.e., a MAPKKKK) that is required for hyphal formation under some conditions (Leberer et al., 1996). Cla4 is also required for hyphal growth; *cla4* mutants produce short malformed germ tubes that fail to grow into long hyphae (Leberer et al., 1997). Both Ste20 and Cla4 possess CRIB domains that mediate interactions with Cdc42 that are necessary for the yeast-to-hyphal switch in *C. albicans* (Su et al., 2007). As in *C. albicans*, *A. gossypii cla4* mutants produce short hyphae that fail to undergo the transition to rapid hyphal growth and apical branching (Ayad-Durieux et al., 2000). In addition, *A. gossypii* Cla4 localization to hyphal tips correlates with this transition. Thus, in the Saccharomycotina, Cla4 is not needed per se for the formation of polarized hyphae, but hyphae cannot achieve their maximal extension rates in its absence. Recent results suggest that a hyphal tip-localized paxillin homologue (i.e., Pxl1) might be a Cla4 substrate that mediates hyphal maturation in *A. gossypii* (Knechtle et al., 2008).

In other filamentous fungi, Cla4 homologues have also been shown to affect hyphal morphogenesis. Deletion of *C. purpurea cla4* prevents the formation of a stable axis of hyphal polarity and results in compact, hyperbranched mycelial colonies (Rolke and Tudzynski, 2008). In this fungus, as well as in *U. maydis*, compelling evidence suggests that Cla4 is a downstream effector of Rac1 and that the Cla4 CRIB domain mediates their interaction (Mahlert et al., 2006; Rolke and Tudzynski, 2008). In other fungi (e.g., *M. grisea* and *C. neoformans*), Cla4 does not have as important a role in maintaining hyphal polarity but is involved in morphogenetic processes such as hyphal branching and cell fusion (Li et al., 2004; Nichols et al., 2004). In these cases, the "division of labor" may be more equitably split between the two PAKs, such that polarized hyphal growth would only be severely compromised by simultaneous deletion of both *cla4* and *ste20*. Nevertheless, Ste20 alone does regulate polarity maintenance in *C. neoformans* as well as spore germination in *P. marneffei* (Nichols et al., 2004; Boyce and Andrianopoulos, 2007). Ultimately, it seems likely that no common theme will emerge for the respective roles of different PAKs in hyphal morphogenesis. Although PAK activity is undoubtedly necessary for polarized hyphal growth, the relative functions of Ste20 and Cla4 in a given filamentous fungus presumably reflect its lifestyle and evolutionary history.

In the model yeasts, NDRs regulate mitotic exit (*S. cerevisiae* Dbf2), septum formation (*S. pombe* Sid2), and polarized growth (*S. cerevisiae* Cbk1 and *S. pombe* Orb6) (summarized by Hergovich et al., 2006). Though numerous genetic and physical interactions have been documented, the mechanisms by which yeast NDRs affect polarized growth are not known. One possibility, as recently suggested for *S. pombe* Orb6, is regulation of Cdc42 GAPs (Das et al., 2007). Far less is known about the roles of NDR kinases in hyphal morphogenesis, though one family member, Cot1 (=Cbk1/Orb6) has been implicated in polarized hyphal growth in several filamentous fungi (e.g., *N. crassa*, *A. nidulans*, *C. trifolii*, and *C. purpurea* [Yarden et al., 1992; Chen and Dickman, 2002; Scheffer et al., 2005b; Johns et al., 2006]). In these fungi, deletion of Cot1 results in the loss of hyphal polarity and triggers excessive formation of lateral branches. *N. crassa* COT-1 has been localized to the plasma membrane as well as to the cytoplasm, where it exhibits a vesicular/reticulate localization pattern (Gorovits et al., 2000; Seiler et al., 2006). A somewhat similar staining pattern was observed for *A. nidulans* Cot1, with occasional localization to the hyphal tip also noted (Johns et al., 2006). Detailed genetic analyses of *N. crassa* have shown that COT-1 functionally interacts with POD-6, a member of the GCK subfamily of Ste20 kinases (i.e., Ste20 kinase homologues with no CRIB domain) (Seiler et al., 2006). Furthermore, this interaction apparently contributes to polarized hyphal growth in a dynein/kinesin-dependent manner, because localization of both COT-1 and POD-6 is disrupted by mutations affecting microtubule motors. In addition, the loss of dynein function readily suppresses defects caused by *cot-1* mutations in *N. crassa* (Bruno et al., 1996b). When considered in light of the known functions and interactions of *S. pombe* Orb6, the characterization of *N. crassa* COT-1 seems to suggest that NDR kinases may participate in defining the zone of polarized growth at hyphal tips. However, further investigation in a broader range of filamentous fungi will be needed to obtain a deeper understanding of how these kinases regulate polarized hyphal growth. In particular, it will be important to determine how NDR and PAK kinases function relative to each other as well as to the monomeric GTPases that regulate hyphal morphogenesis.

Positional Markers

Polarized hyphal growth can be distilled down to a relatively simple task: organize the morphogenetic machinery so that vesicles carrying the components needed for cell surface expansion and cell wall deposition are targeted to a discrete

location that defines the hyphal tip. Although several monomeric GTPases and protein kinases have been implicated in the localized recruitment of the morphogenetic machinery, a key unanswered question is how these signaling components are locally activated. In other words, what is the nature of the positional markers, if any, that define the hyphal tip? Perhaps the best-known positional markers in fungi are those that specify the pattern of bud site selection in *S. cerevisiae* (Chant, 1999; Chang and Peter, 2003). However, with a few notable exceptions (Bud3 and Bud4/Int1), these markers are not that well conserved in organisms other than yeast and its immediate relatives (Gale et al., 2001; Wendland, 2003; Harris and Momany, 2004). Thus, filamentous fungi presumably employ an alternate mechanism to generate positional signals at the hyphal tip. At this time, there appear to be two reasonable possibilities. First, homologues of the complex used to mark cell ends in *S. pombe* are conserved in *A. nidulans* and are necessary for directed hyphal extension (Takeshita et al., 2008). In this complex, TeaR (= *S. pombe* Mod5) functions as a membrane-associated receptor for TeaA (= *S. pombe* Tea1), which is delivered on the plus end of microtubules and likely facilitates localized actin filament formation by recruiting the formin SepA. If the formation of this complex is disrupted, hyphae exhibit meandering growth because of failure to properly position the Spitzenkörper at the hyphal tip. Furthermore, the localization of both TeaR and TeaA at hyphal tips is dependent upon the presence of intact sterol-rich membrane domains (SRDs). Previous results show that these domains, which are defined by filipin staining and presumably represent aggregates of sphingolipid- and sterol-rich lipid rafts, localize to hyphal tips and are needed for polarized hyphal growth (Martin and Konopka, 2004; Pearson et al., 2004; Li et al., 2006; Alvarez et al., 2007). Collectively, these observations suggest an attractive mechanism whereby SRDs confined to the hyphal tip are able to locally organize the actin and microtubule cytoskeleton to maintain a stable axis of hyphal growth. Nevertheless, it remains to be determined how the SRDs are themselves generated in the correct location. In addition, because *teaR* and *teaA* mutants can still produce polarized hyphae, this mechanism cannot account for the initial establishment of a polarity axis during spore germination or hyphal branching.

A second possible mechanism for the positional marking of polarized growth sites in fungal hyphae is the use of tip high gradients of calcium and/or ROS (Jackson and Heath, 1993; Semighini and Harris, 2008). The likely role of these gradients in enforcing apical dominance presumably reflects their ability to mark the hyphal tip as the zone of active growth. By analogy to *Arabidopsis* root hairs, calcium and ROS could conceivably generate interdependent gradients that promote calcium influx only at the apex of the hyphal tip (Takeda et al., 2008). Because of its ability to modulate Cdc42 signaling (i.e., by binding to the GEF Cdc24 [Miyamoto et al., 1991]), calcium could subsequently trigger monomeric GTPase signals that locally recruit and activate the morphogenetic machinery.

It is also worth noting that positional landmarks may not be needed for the establishment of hyphal polarity or even for its subsequent maintenance. Whereas yeast cells possess obvious landmarks that can provide a template for a new polarity axis (for example, bud scars or birth scars in *S. cerevisiae* or the old end of *S. pombe* cells), no such marker has yet been identified for a filamentous fungus. Furthermore, although fungal hyphae display well-known tropisms (i.e., thigmotropism and galvanotropism [Brand et al., 2007]), examples of environmental cues that generate a polarity axis during spore germination or lateral branching have not yet been described. This does not imply that such a phenomenon does not exist, as, for example, the host surface undoubtedly plays some sort of role in directing the axis of spore polarization in plant pathogenic fungi (for an example, see Hardham, 2001). Nevertheless, there is sufficient reason to believe that spores undergoing isotropic expansion can generate a polarity axis at a random site. Clear precedence for this has emerged from studies of *S. cerevisiae*, where intertwined feedback loops can amplify initially stochastic fluctuations in Cdc42 levels to break cellular symmetry in the absence of any known positional landmark (Ozbudak et al., 2005). A key premise underlying "stochastic polarization" in yeast is the coupling of localized exocytosis at the polarization site to endocytosis in flanking regions (Wedlich-Soldner et al., 2003; Marco et al., 2007). Because the recently described tip growth complex of *A. nidulans* essentially provides a mechanism for accomplishing this (Taheri-Talesh et al., 2008), the maintenance of a stable polarity axis in rapidly extending hyphae may not necessarily require the presence of a positional landmark. If a similar complex exists on germinating spores (the tip growth complex has been observed only in mature hyphae), the initial establishment of hyphal polarity may also be independent of any landmark.

When considering the identity of positional markers that might influence the specification of a polarity axis during hyphal morphogenesis, the possible roles of nuclei cannot be ignored. Although spore polarization is apparently not dependent upon nuclear division in filamentous fungi (for an example, see Harris, 1999), anecdotal observations do suggest that nuclei are capable of determining the number and position of germ tubes that emerge from swollen spores. For example, a handful of *A. nidulans* mutants produced large multinucleate spores that eventually yielded germ tubes (i.e., *fimA* and *swoA* mutants shifted to permissive temperature [Momany et al., 1999; Upadhyay and Shaw, 2008]). Notably, when these spores polarize, they often generate multiple germ tubes that emerge simultaneously. However, if nuclear division is blocked, the resulting swollen uninucleate spores produce only a single germ tube (H. Tedford and S. D. Harris, unpublished observations). It has also been demonstrated that nuclear signals play a critical role in specifying septation sites in *A. nidulans* (Wolkow et al., 1996). Thus, nuclei could potentially facilitate the generation of a positional landmark that specifies a polarity axis. One context in which this might be important is in spores that have already produced one germ tube. In this case, the second germ tube is highly biased towards the opposite hemisphere of the spore (i.e., the bipolar germination pattern [Harris et al., 1999]). Assuming that nuclear division precedes the emergence of the second germ tube, astral microtubules emanating from the poles of a mitotic spindle aligned along the primary polarity axis could conceivably deposit a membrane marker that ensures the second germ tube emerges at a ~180° angle relative to the first.

SRDs

The possible role of SRDs in the regulation of polarized hyphal growth has drawn considerable interest over the past few years. As previously noted, these domains are defined by filipin staining and presumably represent aggregates of sphingolipid- and sterol-rich lipid rafts that localize to hyphal tips (Alvarez et al., 2007). The importance of lipid rafts in highly polarized growth has been amply documented in animals (e.g., neurons [Guirland et al., 2004]). SRDs have also been described in the model yeasts and implicated

in the formation of polarized mating projections (Proszynski et al., 2006) as well as in cytokinesis (Takeda et al., 2004). Their relevance to polarized hyphal growth is based upon two sets of observations. First, SRDs localize to hyphal tips in several filamentous fungi, including *C. albicans*, *C. neoformans*, *A. nidulans*, and *Fusarium graminearum* (note that, at least in *A. nidulans*, they also localize to septa) (Martin and Konopka, 2004; Nichols et al., 2004; Pearson et al., 2004; Rittenour and Harris, 2008). Second, disruption of SRD organization and/or formation destabilizes existing polarity axes and likely blocks the formation of new ones (Cheng et al., 2001; Martin and Konopka, 2004; Pearson et al., 2004; Li et al., 2006). These effects are triggered by chemical treatments (myriocin, filipin, and ketoconazole) and mutations (e.g., *A. nidulans aurA*, *mesA*, *barA*, and *basA*) that affect the synthesis of sphingolipids and sterols. Because these same treatments and mutations also disrupt the organization of the actin cytoskeleton at hyphal tips (Pearson et al., 2004; Li et al., 2006), it seems likely that SRDs function in some manner to regulate actin filament formation. As characterized in *A. nidulans*, one potential mechanism by which SRDs presumably accomplish this is by recruitment of the TeaR-TeaA complex to hyphal tips, which in turn promotes localization of the formin SepA (Takeshita et al., 2008). Nevertheless, other fungal proteins known to associate with lipid rafts (i.e., GPI-anchored proteins and superoxide dismutase [Siafakas et al., 2006]) are likely components of SRDs, where they too could influence polarized hyphal growth by regulating recruitment of the morphogenetic machinery.

Septins

The septins are GTP-binding proteins that form higher-order structures involved in diverse aspects of cellular morphogenesis (Weirich et al., 2008). They were first identified and characterized in *S. cerevisiae*, where they are required for cytokinesis and regulate bud morphology (Longtine et al., 1996). Subsequent studies have shown that the septins are broadly conserved in fungi and animals. They can be assigned into seven distinct subgroups, five of which are represented in fungi, including one that appears to be specific to the filamentous fungi (Pan et al., 2007). The role of septins in polarized hyphal growth remains an area of active investigation, but insights have emerged from several different studies (Douglas et al., 2005). During the yeast-to-hyphal transition in *C. albicans*, a septin band forms at the base of the emerging germ tube and a septin cap appears at its tip (Warenda and Konopka, 2002). As the germ tube elongates, these structures then disappear prior to the organization of a septin collar within the germ tube itself. Ultimately, the collar splits into two rings, between which the primary septum is deposited. Genetic analyses demonstrate that two of the *C. albicans* septins (Cdc3 and Cdc12) are essential, whereas deletion of two others (Cdc10 and Cdc11) adversely impacts hyphal morphogenesis (Warenda and Konopka, 2002). Based on these observations, it seems likely that the septin band facilitates germ tube emergence possibly by marking the polarization site and recruiting the morphogenetic machinery. Once the germ tube has formed, the septin ring is required for septum formation. Notably, the fifth *C. albicans* septin, Sep7, controls the stabilization of the septin ring and prevents the recruitment of components needed for septum degradation (*sep7* mutants form hyphae in which cells separate due to degradation of the primary septum [Gonzalez-Novo et al., 2008]). Additional observations implicate the septin ring in the regulation of hyphal extension. Prior to formation of the ring, the septin cap likely directs exocytosis to the hyphal tip in a process mediated in part by binding of Cdc11 to the exocyst component Sec3 (Li et al., 2007; Sinha et al., 2007). Once the cap disappears and the septin ring forms, Cdk-dependent (i.e., Ccn1-Cdc28) phosphorylation of Cdc11 weakens its affinity for Sec3, thereby ensuring that the exocyst continues to target exocytosis to the hyphal tip and not to the incipient septation site. This transition may in fact mark the point of hyphal maturation in *C. albicans*.

Compared to *C. albicans*, much less is known about septin function in other filamentous fungi. *A. gossypii* septins localize to the hyphal tip and branch sites as discontinuous rings (Helfer and Gladfelter, 2006). Mitosis occurs in an asynchronous manner in *A. gossypii* (Gladfelter, 2006a), and septin ring localization appears to be a key determinant that specifies zones of mitotic activity (Helfer and Gladfelter, 2006). This is based on the observations that mitotic nuclei are predominantly found adjacent to septin rings and that mitosis occurs at random sites in a septin mutant. By providing a scaffold for the localized recruitment of factors that promote mitosis, the septins presumably ensure a ready supply of nuclei to support the extension of active hyphal tips in *A. gossypii*. A correlation between septin localization and lateral branching has also been noted in *A. nidulans* (Westfall and Momany, 2002). In this case, the septin AspB (=Cdc3) exhibits premitotic localization to incipient branch sites and persists at the base of the branch, while adjacent nuclei enter mitosis. The ability of septins to coordinate branching with mitosis in divergent filamentous fungi that employ different modes of mitosis (i.e., asynchronous versus parasynchronous) suggests that this is a universal feature of these proteins. Genetic analysis of *A. nidulans* and *U. maydis* has revealed additional roles for septins in hyphal morphogenesis and septum formation, as well as in developmental processes (Westfall and Momany, 2002; Boyce et al., 2005b).

Although a great deal remains to be learned about the function of septins in polarized hyphal growth, they rightfully deserve their label as crucial determinants of cell morphology in filamentous fungi (Gladfelter, 2006b). As scaffolds capable of recruiting multiple regulators, they seemingly play a key role in the temporal and spatial coordination of both branch formation and septation with mitosis. In addition, based on their known functions in *S. cerevisiae*, septins could conceivably promote compartmentalization of the cell surface at hyphal tips (Lindsey and Momany, 2006). For example, they might act as "fences" to define the boundaries of SRDs. Finally, limited evidence already suggests that septins likely determine patterns of hyphal morphogenesis. This includes their apparent role in regulating the maturation of *C. albicans* hyphae (Sinha et al., 2007). Moreover, in *A. nidulans*, the asymmetric localization of AspB on the apical side of completed septa raises the possibility that they act as markers that specify the overall polarity of a growing hypha (Westfall and Momany, 2002). Future studies will undoubtedly uncover additional regulatory functions for septins in fungal hyphae. Nevertheless, it will soon be necessary to begin the search for relevant septin-associated proteins that mediate these functions.

ROLES OF THE SPITZENKÖRPER AND POLARISOME IN POLARIZED HYPHAL GROWTH

Localization and Composition of the Polarisome

In *S. cerevisiae*, a group of proteins consisting of Spa2, Bud6, and Pea2 localize to a cell surface crescent at the incipient

bud site and cosediment as a 12S complex upon sucrose density gradient centrifugation (Sheu et al., 1998; summarized by Sudbery and Court, 2007). This complex, which was termed the polarisome, is thought to mediate the formation of actin filaments by the formin Bni1 in response to Cdc42 signals. Besides activating Bni1 at the incipient bud site, Cdc42 has also been implicated in the recruitment of Spa2 (Rida and Surana, 2005). In turn, both Spa2 and Bud6 are known to facilitate Bni1 function; Spa2 promotes stable localization of Bni1, whereas Bud6 stimulates Bni1 actin-nucleating activity (Ozaki-Kuroda et al., 2001; Sagot et al., 2002). The function of Pea2 relative to Cdc42 and Bni1 remains a mystery. In addition to their roles in the regulation of actin filament formation, Spa2 and Bud6 are involved in other aspects of cellular morphogenesis in *S. cerevisiae*. For example, Spa2 interacts with numerous proteins, including the MAP kinase Slt2, the Rab GAPs Msb4/Msb4, and the Cdc42 effectors Gic1/Gic2 (for examples, see Sheu et al., 1998; Jaquenoud and Peter, 2000; van Drogen and Peter, 2002; and Tcheperegine et al., 2005), thereby suggesting that it provides a scaffold for multiple steps of cellular morphogenesis. Bud6 has an additional role in microtubule capture at the cell cortex (Segal et al., 2000). These functions are presumably independent of Spa2 and Bud6 association with each other in the context of the polarisome, though this has not been demonstrated. Accordingly, the polarisome may consist of additional components that transiently interact with these core components.

The conservation of the polarisome in other fungi is a subject that retains considerable interest. Homologues of Spa2, Bud6, and Bni1 have been characterized in *C. albicans*, *A. gossypii*, and *A. nidulans*. At this time, a general consensus for the function or localization of the polarisome in growing hyphae has yet to emerge. In *C. albicans*, both Spa2 and Bud6 localize to a surface cap at hyphal tips that likely corresponds to the polarisome, whereas Bni1 appears as a spot that subtends the cap (Crampin et al., 2005; Martin et al., 2005). As outlined in more detail below, growing evidence suggests that this spot is in fact the Spitzenkörper. Deletions of *spa2*, *bud6*, or *bni1* all lead to similar morphological defects and a failure to maintain hyphal polarity (Zheng et al., 2003; Li et al., 2005; Martin et al., 2005; Song and Kim, 2006). Somewhat similar localization patterns are seen in *A. nidulans* (Sharpless and Harris, 2002; Virag and Harris, 2006); SepA (=Bni1) localizes to the surface cap and the subtending spot, whereas SpaA (=Spa2) appears as a small surface crescent (an identical SpaA localization pattern was reported in *A. niger*, as well; Meyer et al., 2008). Deletion of *sepA* or *spaA* compromises the maintenance of hyphal polarity, though the defects are far more severe in *sepA* mutants (Sharpless and Harris, 2002; Virag and Harris, 2006). Notably, unlike what occurs in *S. cerevisiae*, SepA localization is not dependent upon SpaA in *A. nidulans* hyphae. In *A. gossypii*, Bni1 forms a crescent at the surface of hyphal tips that does not appear to overlap with Spa2, which localizes to a central spot just behind the tip (Knechtle et al., 2003; Schmitz et al., 2006). Genetic analyses demonstrate that Bni1 is absolutely essential for the establishment of hyphal polarity in *A. gossypii*. By contrast, Spa2 is needed for hyphae to attain normal extension rates and to generate the proper branching pattern.

What, then, can be inferred about the function and localization of the polarisome in fungal hyphae? First, it is not yet apparent that the polarisome, as defined in *S. cerevisiae*, even exists. Spa2 and Bni1 do not display extensive colocalization in those filamentous fungi that have been examined, and as observed in *A. nidulans*, formin recruitment may occur independent of Spa2 (Crampin et al., 2005; Philippsen et al., 2005; Virag and Harris, 2006). Less is known about Bud6, but limited evidence from *A. nidulans* suggests that Bud6 and formins are likely to be functional partners (Virag and Harris, 2006). Ultimate proof for the conservation of the polarisome in filamentous fungi will require biochemical and proteomic evidence similar to that used to reveal its existence in *S. cerevisiae*. At the very least, it seems plausible that an analogous structure is present as a surface cap at hyphal tips, where it mediates formin-dependent nucleation of actin filaments in response to Cdc42 signals.

Relationship between the Spitzenkörper and Polarisome

The localization of the *A. nidulans* formin SepA to a dynamic spot just behind the hyphal tip strongly suggested that it might be a component of the Spitzenkörper (Sharpless and Harris, 2002). This was subsequently confirmed when it was shown that SepA colocalizes with the FM4-64-labeled spot that is typically used to define the Spitzenkörper (Harris et al., 2005). Parallel studies with *C. albicans* demonstrated that the formin Bni1 and the myosin regulatory light chain Mlc1 also localized to the Spitzenkörper (Crampin et al., 2005). These observations are consistent with the notion that the Spitzenkörper functions as an actin-organizing center.

The localization of SepA to both the polarisome (surface cap) and the Spitzenkörper (subtending spot) suggests a possible functional relationship between these entities. Even in *C. albicans*, where they are spatially distinct objects that coexist in hyphae, but not in yeast cells or pseudohyphae (where the Spitzenkörper is not found), it was still suggested that the Spitzenkörper could plausibly be a "hyperactive" polarisome (Sudbery and Court, 2007). One possible explanation for their relationship is based on the mechanisms underlying formin-mediated nucleation of actin filaments. The *S. pombe* formin For3 nucleates short filaments at the cell tip that are then released and carried into the cell interior along actin cables (Martin and Chang, 2006). These movements are dependent upon the presence of actin filaments and require the C-terminal FH2 domain of For3. Based on this precedent, it is conceivable that the SepA spot could represent an accumulation of formins that have been transported along actin filaments that originated from the cell surface cap. In accord with this idea, the SepA spot disappears when actin filaments are eliminated by treatment with cytochalasin A, whereas localization at the cell surface remains (Sharpless and Harris, 2002). In addition, the C-terminal half of SepA, which houses the FH2 domain implicated in actin nucleation, is required for SepA localization to the spot (Sharpless and Harris, 2002).

Perhaps it is more appropriate to view the polarisome and Spitzenkörper as components of the larger tip growth apparatus (Taheri-Talesh et al., 2008). Localization of the polarisome, consisting at the very least of a formin plus Bud6, to the surface cap may ensure the formation of actin filaments that are used to transport exocytic vesicles stored in the Spitzenkörper. Formins delivered to the Spitzenkörper along the same actin filaments may be recycled back to the cap by diffusion or by transport along microtubules. Other multiprotein complexes implicated in localized exocytosis within the tip growth apparatus (i.e., the exocyst) may also display colocalization with the Spitzenkörper, depending on the dynamics of their individual components. The functional relationships between these complexes and the Spitzenkörper may be choreographed by Cdc42, which

also localizes to the surface cap in *A. nidulans* (note that the polarisome and exocyst are known Cdc42 effectors).

EVOLUTION OF MORPHOGENETIC MECHANISMS IN FUNGI

Conservation of Genes Required for Highly Polarized Growth

The availability of a large number of annotated fungal genome sequences has ushered in a new era whereby comparative genomic approaches can be employed to make inferences regarding the evolution of morphogenetic mechanisms in fungi. A particularly interesting issue is the relationship between highly polarized cell types in fungi (i.e., hyphae) and their opisthokont relatives in the animals (i.e., neurons and other migratory cells). Does this mode of growth require specific adaptations to the morphogenetic and/or regulatory machinery that are not required in less polarized cell types? A cursory comparison of the genes implicated in polarized hyphal growth with those needed for polarized morphogenesis of yeast cells suggests that this might be true. For example, filamentous fungi possess extra GTPase modules (e.g., Rac1) and microtubule motor proteins (e.g., kinesins) that are involved in polarized hyphal growth and are either absent or poorly conserved in yeast but are well conserved in neurons (for examples, see Harris and Momany, 2004; and Schuchardt et al., 2005). Additional examples include the role of ROS generated by Nox complexes in the maintenance of apical dominance in hyphae and neurons (Ibi et al., 2006; Semighini and Harris, 2008). Whether these examples reflect selection for mechanisms that enable highly polarized growth remains to be determined. Nevertheless, a more systematic analysis that is informed by bioinformatics approaches would seem to be warranted. From a practical viewpoint, this would reveal the extent to which genetically tractable model filamentous fungi such as *A. nidulans* and *N. crassa* could be used to model neurodegenerative disorders such as ataxia telangiectasia (Malavazi et al., 2006).

The Origins of Polarized Hyphal Growth

It is widely accepted that the Chytridiomycetes represent the most ancestral fungal phylum (James et al., 2006). Chytrids are largely aquatic fungi that produce motile zoospores that are capable of amoeboid movement (Longcore at al., 1999). In addition, certain chytrids (i.e., the monocentric chytrids) do not produce hyphae and in many respects appear to be morphologically similar to animal cells (Taylor et al., 2004). The Zygomycetes represent another basal fungal phylum that likely originated on multiple separate occasions from the chytrids (James et al., 2006). Notably, the Zygomycetes are terrestrial fungi that grow solely by hyphal extension (though they share certain polarized cell types with chytrids, such as rhizoids). Thus, at face value, it would appear that early fungi exhibited diverse morphologies that bear some similarity to their closest animal relatives in the Protozoa. However, the commitment to a terrestrial lifestyle may have selected for polarized hyphae as the dominant form of growth. The recent completion of annotated genome sequences for a chytrid, *Batrachochytrium dendrobatidis* (http://www.broad.mit.edu/annotation/genome/batrachochytrium_dendrobatidis), and two zygomycetes (*Rhizopus oryzae* and *Phycomyces blakesleeanus* [http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/MultiHome.html and http://genome.jgi-psf.org/Phybl1/Phybl1.home.html]) should make it possible to use comparative genomic approaches to test this hypothesis regarding the origins of hyphal growth. Two relevant observations have already emerged from a preliminary annotation of these genome sequences (S. Harris, unpublished results). First, the *B. dendrobatidis* genome possesses reasonably well-conserved homologues of proteins implicated in animal cell migration (i.e., talin, vinculin, and parvin) that are missing from *R. oryzae* or *P. blakesleeanus*. Second, despite their ancestral position in the fungal phylogeny, the chytrids and zygomycetes appear to exhibit increased complexity in terms of the gene content devoted to morphogenesis. For example, whereas *A. nidulans* and *N. crassa* each possess only a single formin, *R. oryzae* possesses at least eight distinct formin homologues. Furthermore, *R. oryzae* harbors increased numbers of annotated Ras/Rho-related GTPases (70 versus 27), Rho GEFs (23 versus 5), and Rho GAPs (34 versus 7) compared to *A. nidulans*. Although numerous explanations for this paradox are possible, these differences suggest that commitment to polarized hyphal growth might have introduced a "morphological bottleneck" that ultimately triggered the loss of genes needed for alternate forms of growth.

What Are the Minimal Requirements for a Functional Hypha?

The use of polarized hyphae as means of vegetative propagation is not a unique attribute of the filamentous fungi. Organisms within the animal (e.g., oomycetes) and bacterial (e.g., streptomycetes) kingdoms generate branched hyphae that closely resemble those produced by fungi. Additional cell types in plants (e.g., moss protonema) also bear considerable morphological similarity to hyphae. Accordingly, hyphal growth is likely a convergent solution to the common problem of nutrient acquisition across variable habitats. This raises an interesting question: is it possible to define a minimal set of functions required for polarized hyphal growth? It seems likely that components of the morphogenetic machinery that enable localized cell surface expansion and cell wall deposition would be universally important for hyphal growth. For example, even in Streptomyces, the tropomyosin-like protein DivIVA is required for normal hyphal morphogenesis (Fiardh, 2003). However, a more interesting challenge will be to determine whether regulatory functions, such as GTPases, as well as proteins involved in calcium and ROS signaling, are also universal components of the minimal gene set needed for hyphal growth.

The comparative analysis of morphogenesis in *S. cerevisiae* and *A. gossypii* provides potentially informative insight into the minimal requirements for polarized hyphal growth. Although these fungi are closely related members of the Saccharomycotina, *S. cerevisiae* grows as yeast and pseudohyphae (though it is capable of forming hypha-like filaments in response to mating pheromone), whereas *A. gossypii* is capable of hyphal growth only. This difference in growth form does not reflect large differences in their respective gene sets, as ~95% of *A. gossypii* genes are orthologues of *S. cerevisiae* genes (summarized by Philippsen et al., 2005). As might be expected, genes that are obviously important for polarized growth, such as Cdc24, Cdc42, Rho1, and Rho3, are as important for hyphal growth in *A. gossypii* as they are for morphogenesis in *S. cerevisiae*. However, genetic analyses have also identified homologues of *S. cerevisiae* genes that have strikingly different morphological phenotypes when deleted in *A. gossypii*. For example, despite the presence of two Bnr1 homologues, deletion of Bni1 is lethal in *A. gossypii* due to a failure in polarity establishment, whereas the analogous *S. cerevisiae* mutant is viable and capable of forming a bud (Schmitz et al., 2006). This difference presumably reflects a greater role for formin-nucleated actin cables in supporting

hyphal growth. Other examples of genes with seemingly different roles in polarized hyphal growth compared to their established function in yeast budding include the Ras GTPase Rsr1, the polarisome component Spa2, the PAK kinase Cla4, and the paxillin Pxl1 (Ayad-Durieux et al., 2000; Knechtle et al., 2003; Bauer et al., 2004; Knechtle et al., 2008). Based on these results, it seems possible that *A. gossypii* achieves hyphal growth by merely rewiring the genetic networks that underlie cellular morphogenesis in *S. cerevisiae*. According to this hypothesis, significant changes in gene content might not be necessary to generate polarized hyphae from a nonhyphal cell type.

The analysis of hyphal morphogenesis in *A. gossypii* highlights one additional puzzle. *A. nidulans* and *N. crassa* possess many genes that have critical roles in polarized hyphal growth (e.g., *A. nidulans mesA* and *barA* and *N. crassa pod-2* and *pod-3*) and that are either absent or poorly conserved in *A. gossypii* (Seiler and Plamann, 2003; Pearson et al., 2004; Li et al., 2006). However, in contrast to the mutant phenotypes in *A. nidulans* or *N. crassa*, the absence of these genes in *A. gossypii* in no way limits the ability of this fungus to generate hyphae capable of impressive extension rates. Other examples of functions that are required for efficient hyphal extension in *A. nidulans* but are apparently dispensable in *A. gossypii* include components of the TeaA/TeaR and Nox complexes (Takeshita et al., 2008; Semighini and Harris, 2008). The dispensability of these functions in *A. gossypii* implies that they are not part of the minimal gene set required for polarized hyphal growth. On the other hand, the importance of these functions in *A. nidulans*, and presumably other members of the Pezizomycotina, suggests that they confer some sort of selective advantage. It is not clear what this might be, but it might relate to the ability to form lateral branches, engage in hyphal anastomosis, or produce complex developmental structures.

SUMMARY AND MODELS

The importance of understanding the molecular mechanisms underlying polarized hyphal growth cannot be understated. Because filamentous fungi continue to pose a significant threat to human health and food security, there is considerable interest in the identification of novel targets that will limit fungal growth and thus the capacity to cause disease. As an essential function required for any filamentous fungus to colonize a human or plant host, specific features of polarized hyphal growth will likely provide attractive targets for this purpose. In addition, fungal hyphae share their highly polarized lifestyle with neurons and other migratory cells. It seems likely that at some level, the mechanisms that support this lifestyle will also be shared. The genetic tractability of filamentous fungi such as *A. nidulans* and *N. crassa* affords a tremendous opportunity to elucidate these mechanisms and to acquire insight that might be relevant to neurological disorders and other motor diseases.

Up to now, most of what is known about polarized hyphal growth has been obtained through the use of complementary genetic and microscopic approaches (summarized by Harris et al., 2005). The use of increasingly sophisticated microscopy techniques has revealed the subcellular organization of hyphal tip cells and, in particular, emphasized the role of the Spitzenkörper in polarized hyphal growth. The recent characterization of the hyphal tip apparatus in *A. nidulans* provides an outstanding example of the power of this approach (Taheri-Talesh et al., 2008). The identification and genetic analysis of mutants defective in hyphal morphogenesis date back to the original *Neurospora* mutant screens of Beadle and Tatum (summarized by Mishra, 1977). Over the intervening years, a sizable number of morphological mutants have been characterized, not only in the model fungi *A. nidulans* and *N. crassa*, but also in human and plant pathogens (summarized by Harris, 2006). Originally, these mutants were primarily identified using forward genetics, whereby mutants were generated and subjected to detailed phenotypic analysis before the affected genes were cloned by complementation. The availability of annotated genome sequences combined with the advent of functional genomic tools has, at least in the short term, triggered greater use of reverse genetic approaches aimed at mutating homologues of genes implicated in the polarized growth of yeasts or animal cells.

What has been learned so far? Perhaps the least surprising lesson is that polarized hyphae are not a modified yeast cell (unlike pseudohyphae). Genetic analyses of *N. crassa* and *A. nidulans* have generated a long list of genes needed for hyphal morphogenesis; in many cases, *S. cerevisiae* homologues of these genes, if present at all, have no known role in polarized growth (Harris and Momany, 2004; Harris 2006; Harris et al., 2009). Even *A. gossypii* possesses genes that are required for polarized hyphal growth yet are dispensable in *S. cerevisiae* (for example, Bni1 [Schmitz et al., 2006]). These differences presumably reflect the greater demands that highly polarized growth places on the morphogenetic machinery in hyphae compared to yeast cells (e.g., the long-range transport of vesicles and organelles along microfilament and microtubule tracks). Accordingly, given the current emphasis on the use of reverse genetic approaches, it would seem prudent to avoid sole reliance upon the so-called yeast paradigm. For example, homologues of proteins implicated in neuronal morphogenesis or cell migration would be attractive candidates for genetic analysis.

Another notable lesson that has become apparent is the intricately structured organization of the hyphal tip (Taheri-Talesh et al., 2008). Besides the Spitzenkörper, the hyphal tip also houses complexes such as the polarisome and exocyst. There are undoubtedly many additional complexes that localize to the tip, which should perhaps be viewed as a dynamic assemblage of complexes that function in an integrated manner to drive hyphal extension. Furthermore, exocytic vesicle delivery appears to be confined to the extreme apex of the hyphal tip, whereas flanking regions comprise endocytic zones where surface components are retrieved and recycled for subsequent reuse. The striking similarity between the organization of hyphal tips and that of the neurological synapse lends further credence to the notion that neurons and hyphae share common mechanisms for polarized growth.

What should be done next? It has been argued that the first step towards understanding the molecular basis of a specific cellular process is to assemble a "parts list" (for an example, see Pollard, 2003). At this point, many of the components involved in polarized hyphal growth have been identified. Although genetic and proteomic approaches are still needed to identify additional proteins, efforts to organize these components into functional modules must also be initiated. The construction of protein interaction networks (via yeast two-hybrid analysis, affinity purification, or protein complementation analysis) and genetic interaction networks (via synthetic genetic arrays) has yielded unprecedented insight into the functional modules that control cellular morphogenesis in *S. cerevisiae* (Boone at al., 2007). Similar approaches could also be used with *A. nidulans* or *N. crassa* to characterize the interaction networks that underlie polarized hyphal growth. The resulting information

would provide a framework for subsequent attempts to investigate the biochemical mechanisms that drive the establishment and maintenance of hyphal polarity. In addition, comparison of the "morphogenesis networks" from filamentous fungi and yeasts might shed light on how changes in network architecture impact cell shape in fungi.

Another key objective is to obtain a detailed understanding of how the hyphal tip is organized. The continued use of GFP-based probes to characterize protein localization and colocalization patterns within the tip will provide needed insight into the composition and dynamic behavior of the different complexes that comprise the hyphal tip apparatus. In addition, it is likely that the plasma membrane within the tip region is demarcated into distinct domains that differ in sterol and phospholipid composition. The identification of these domains would enable the characterization of their presumptive roles in organizing the tip. Finally, high-resolution electron tomography permits three-dimensional reconstruction of cellular structures at a nanoscale level. This approach has already offered a tantalizing glimpse into the organization of the *A. nidulans* hyphal tip (Harris et al., 2005). It is not unreasonable to envision these "fungal computerized axial tomography scans" providing an exquisitely detailed and quantitative picture of hyphal tip organization. Notably, the use of quantitative microscopy as a tool for the phenotypic analysis of morphological mutants has proven to be an effective approach for a systems-based analysis of morphogenesis in yeast.

Several models have attempted to provide a mechanistic basis for understanding polarized hyphal growth based on previous microscopy-based studies (summarized by Steinberg, 2007). The steady-state model is based on the notion that cell wall polymers are assembled at the immediate hyphal apex but are not cross-linked until they are displaced laterally (Sietsma and Wessels, 1994). Accordingly, the cell wall at the immediate apex remains plastic and responsive to internal turgor pressure, which in turn drives extension of the hypha. A major weakness with this model is the conflicting evidence regarding the necessity of turgor pressure for hyphal extension (Steinberg, 2007). The vesicle supply center model is founded on the role of the Spitzenkörper in polarized hyphal growth (Bartnicki-Garcia, 2002). The Spitzenkörper is thought to serve as a vesicle distribution hub in the hyphal tip. The release of vesicles in a radial manner coupled to the forward movement of the Spitzenkörper generates a gradient of exocytosis that leads to the typical hyphoid shape of filamentous fungi. On the other hand, if the Spitzenkörper remains in a fixed position, ovoid shapes characteristic of yeast cells are produced. Although well supported by experimental observations and mathematical models, recent evidence suggests that certain features of this model may need to be reconsidered (for example, the exocytic zone at the hyphal apex is much smaller than would be predicted by the model [Taheri-Talesh et al., 2008]). It has been suggested that a combination of the steady-state and vesicle supply center models may best account for hyphal morphogenesis (Steinberg, 2007). Nevertheless, the amoeboid model, which posits that forces generated by the cytoskeleton promote hyphal extension, cannot be excluded (Heath and Steinberg, 1999). Notably, *N. crassa* protoplasts can form cytoplasmic extensions that resemble pseudopodia from migrating animal cells (Steinberg, 2007). This observation, coupled with the conservation of a limited number of proteins implicated in animal cell migration, suggests that amoeboid movements might have a previously unforeseen role in hyphal extension.

Steinberg (2007) and Taheri-Talesh et al. (2008) have proposed updated models for polarized hyphal growth that incorporate observations from more recent molecular studies. Key elements of these models include the roles of (i) SRDs in directing the localization of the polarisome at the hyphal tip, (ii) formins in promoting the assembly of actin filaments anchored at the hyphal tip, (iii) myosins in the microfilament-based transport of vesicles to a discrete exocytic zone at the apex of the tip, (iv) actin patch-mediated endocytic retrieval of cell surface components from sites flanking the apex, and (v) microtubule-based transport of vesicles to and from the tip region. Both turgor pressure and forces generated by the cytoskeleton are proposed to drive the tip forward, and the tip itself likely houses a highly structured but dynamic apparatus that coordinates localized exocytosis with endocytosis.

Although these models account for much of what is known about the extension of mature hyphae, they still do not address the key issue of how hyphal polarity is initially established; that is, what is the key event(s) that breaks the symmetry of a swollen spore or a subapical hyphal cell? It might be tempting to consider specific proteins (e.g., proteins analogous to the *S. cerevisiae* bud site selection markers) or membrane domains (e.g., domains such as the SRDs implicated in recruitment of cell end factors in *A. nidulans*)

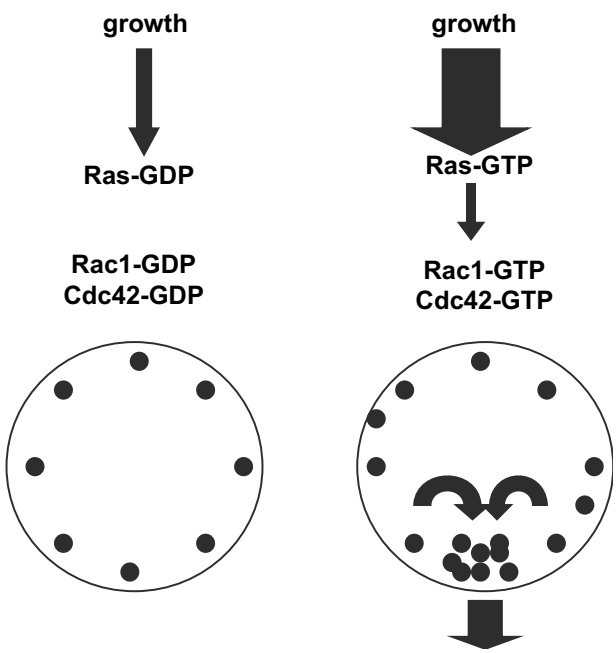

FIGURE 3 Model for the coordination of polarity establishment with growth by monomeric GTPases. (Left) Prior to receiving a strong growth signal (e.g., glucose), Ras is in an inactive GDP state that is unable to activate Rac1 or Cdc42. As a result, the latter GTPases (dots) are uniformly distributed. (Right) Upon reception of a strong growth signal, activated Ras (i.e., Ras-GTP) triggers the activation of Rac1 and Cdc42. Stochastic fluctuations in Rac1-GTP and/or Cdc42-GTP levels lead to local asymmetries in what was initially a uniform distribution. Feedback loops reinforce these asymmetries until a threshold is reached at a given site that then becomes the dominant polarity axis (black arrow). Both endocytic recycling of surface components and enhanced Rac1/Cdc42 GAP activity at nonpolarization sites likely play a key role in reinforcing the polarity axis.

as sources of positional information that initiate a polarity axis, but there is no reason that this event could not simply be a stochastic process. For instance, random fluctuations in local activity of a monomeric GTPase could be amplified by feedback loops, leading to asymmetries in membrane organization that enable recruitment of cell end factors, the polarisome, and other components of the hyphal tip apparatus (Fig. 3). Attractive candidates for the GTPase "trigger" are Rac1 and/or Cdc42. The emerging role of these GTPases as downstream effectors of Ras1 in fungi coupled with their known ability to coordinate the morphogenetic machinery suggests that they may define a signaling nexus that links the commitment to form a polarity axis to growth conditions (Fig. 3). Future experiments that exploit genomic and proteomic tools will undoubtedly provide new insights that test the validity of this hypothesis and reveal the key symmetry-breaking event(s) that lead to polarized hyphal growth.

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19

Hyphal Fusion

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Filamentous fungi in the Ascomycete and Basidiomycete groups grow by hyphal tip extension, branching and fusion to make an interconnected, supracellular network of hyphae that is the hallmark of these organisms (Buller, 1933; Read, 2007). Hyphal anastomosis also occurs in Zygomycetes (Griffin and Perrin, 1960), where it apparently is not a common phenomenon (Gregory, 1984), and in arbuscular mycorrhizal fungi, which are members of the Glomeromycota (Giovannetti et al., 1999, 2001). Species within the Oomycota, which undergo hyphal tip growth and branching but which are more closely related to algae and are members of the Kingdom Stramenopila, also undergo hyphal fusion (Stephenson et al., 1974).

Hyphal fusion occurs at a number of stages during the life cycle of filamentous fungi, particularly during (i) colony initiation, (ii) mature colony development, and (iii) sexual development. A wide range of important roles are performed by hyphal fusion during the life cycle. This chapter focuses on hyphal fusion in filamentous ascomycete and basidiomycete species, with emphasis on the model ascomycete fungus, *Neurospora crassa*. It reviews the different types of hyphal fusion, its mechanistic basis, and the varied functions that it serves, and it compares hyphal fusion with processes of cell fusion in fungi and other eukaryotic species. Hyphal fusion has also been recently reviewed by Glass et al. (2000, 2004), Roca et al. (2005a), Glass and Fleißner (2006), Read and Roca (2006), and Fleißner et al. (2008).

HYPHAL FUSION DURING DIFFERENT STAGES OF THE LIFE CYCLE

Hyphal Fusion During Colony Initiation

Hyphal fusion between spores and spore germlings during colony initiation is very common. It is most often observed between conidia and conidial germlings and has been demonstrated in over 73 ascomycete species (Roca et al., 2005a). Hyphal fusion has also been observed between ascospore germlings and between urediospore germlings (Read and Roca, 2006).

Tulasne and Tulasne (1863) first illustrated fusions between conidia and conidial germlings in a variety of species. An important early experimental study of fusion between germinating conidia was performed by Köhler (1930). He described fusion between germinating conidia of the same or different species via small hyphal bridges ("Fusionshyphen"), which were significantly narrower than germ tubes. Numerous other papers subsequently showed images of fused conidia and conidial germlings in culture (Hay, 1995), in asexual reproductive structures (Mesterhazy, 1973), or on host plants (Latunde-Dada et al., 1999). However, it was not fully appreciated until recently that fusion between conidia/conidial germlings involves the formation and interaction of specialized hyphae, which are different from germ tubes and which have been termed "conidial anastomosis tubes" (CATs) (Roca et al., 2003, 2005a, 2005b).

The initial characterization of CATs was made in the plant pathogen *Colletotrichum lindemuthianum* (Roca et al., 2003) and the saprotrophic species *N. crassa* (Roca et al., 2005b). They were shown to have the following combination of characteristics which distinguishes them from conidial germ tubes.

1. CATs are usually thinner and shorter than germ tubes and exhibit determinate growth. Germ tubes differentiate into vegetative hyphae of the mature colony (Fig. 1A through D) (Araujo-Palomares et al., 2007; Roca et al., 2005b). However, it is often not possible to define CATs on the basis of their width alone, and this is a problem particularly when identifying CATs that arise from germ tube tips (Fig. 1B) or as germ tube branches (Fig. 1C).
2. CATs are unbranched, while germ tubes undergo branching (Araujo-Palomares et al., 2007; Roca et al., 2005b).
3. CATs in *N. crassa* can arise directly from conidia, from germ tube tips or as subapical branches of germ tubes (Roca et al., 2005a, 2005b) (Fig. 1A through D).

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4. CAT induction is dependent on conidial density. In *N. crassa*, the optimum conidial density is $\sim 10^6$ conidia ml^{-1} (Roca et al., 2005b). Germ tube formation in *N. crassa* is not dependent on conidial density over the range of 10^2 to 10^6 conidia ml^{-1} (H.-C. Kuo and N. D. Read, unpublished data).
5. CATs home towards each other, while germ tubes tend to avoid one other. CAT homing has been most unambiguously demonstrated using optical (laser) tweezer micromanipulation (Fig. 1D). When the relative position of two CATs exhibiting chemotropic growth towards each other is changed by micromanipulation with optical traps, both CATs readjust their direction of growth back towards each other to make contact and fuse at their tips (Fleißner et al., 2005; Roca et al., 2005a; Wright et al., 2007).
6. CATs are under separate genetic control from germ tubes. A growing number of mutants have been isolated that are inhibited at specific stages of CAT fusion but undergo conidial germination and germ tube formation (Table 1).
7. CATs function in interconnecting conidial germlings and are believed to allow the young colony to act as a cooperative individual. Germ tubes, on the other hand, function in colony establishment.

How consistent these features of CATs are in other species will need to be carefully assessed in the future. Some species of filamentous fungi seem to lack CATs (for example, Craven et al. reported that *Alternaria alternata* does not form CATs [Craven et al., 2008]). We are not aware of any published illustrations of CAT fusion between conidial germlings in the model species *Magnaporthe oryzae* or *Aspergillus nidulans*. However, CAT formation in some species may require specific conditions (e.g., nutritional factors or conidial density) that are not commonly used experimentally.

Hyphal Fusion in a Mature Colony

A filamentous fungal colony is morphologically complex and contains a variety of different hyphal types (Bistis et al., 2003). Leading hyphae and their branches at the periphery of a colony typically grow outward, exhibit a subapical branching pattern, and tend to avoid each other (Buller, 1933). Hyphal anastomosis does not normally occur in this peripheral zone of an *N. crassa* colony (Hickey et al., 2002). Behind this peripheral zone, hyphal fusion occurs extensively to produce the characteristic interconnected state of the mature colony (Fig. 1E). Hyphal anastomosis is initiated by the formation of specialized "fusion hyphae," which arise as branches from established hyphae and branches in this part of the colony. Although fusion events are frequent within a colony, they are not uniformly distributed. Another type of hyphal fusion also occurs in mature colonies of nematophagous fungi, such as *Arthrobotrys oligospora*, where hyphal fusion is important for the formation of a range of specialized nematode-trapping structures. These traps can take the form of simple rings, constricting rings, or net-like structures (Barron, 1977; Read and Roca, 2006).

In *N. crassa,* vegetative hyphal fusion in a mature colony has been described in detail using time-lapse confocal microscopy (Hickey et al., 2002). The process can be conveniently divided into three phases: precontact, postcontact, and postfusion (Glass et al., 2000, 2004; Hickey et al., 2002; Read and Roca, 2006), as described below.

Precontact Phase

During the precontact phase in *N. crassa*, the fusion hyphae are induced and grow ("home") towards each other. The proximity of fusion hyphae to other hyphae often results in the initiation of new positively chemotropic hyphal tips that are fusion competent (Buller, 1933; Hickey et al., 2002; Köhler, 1929). The fusion hyphae in *N. crassa* can often be recognized as morphologically distinct from other hyphae. They always appear to be hyphal branches and can vary from being short, peg-like structures to reasonably long and often dichotomously branched hyphae (Hickey et al., 2002).

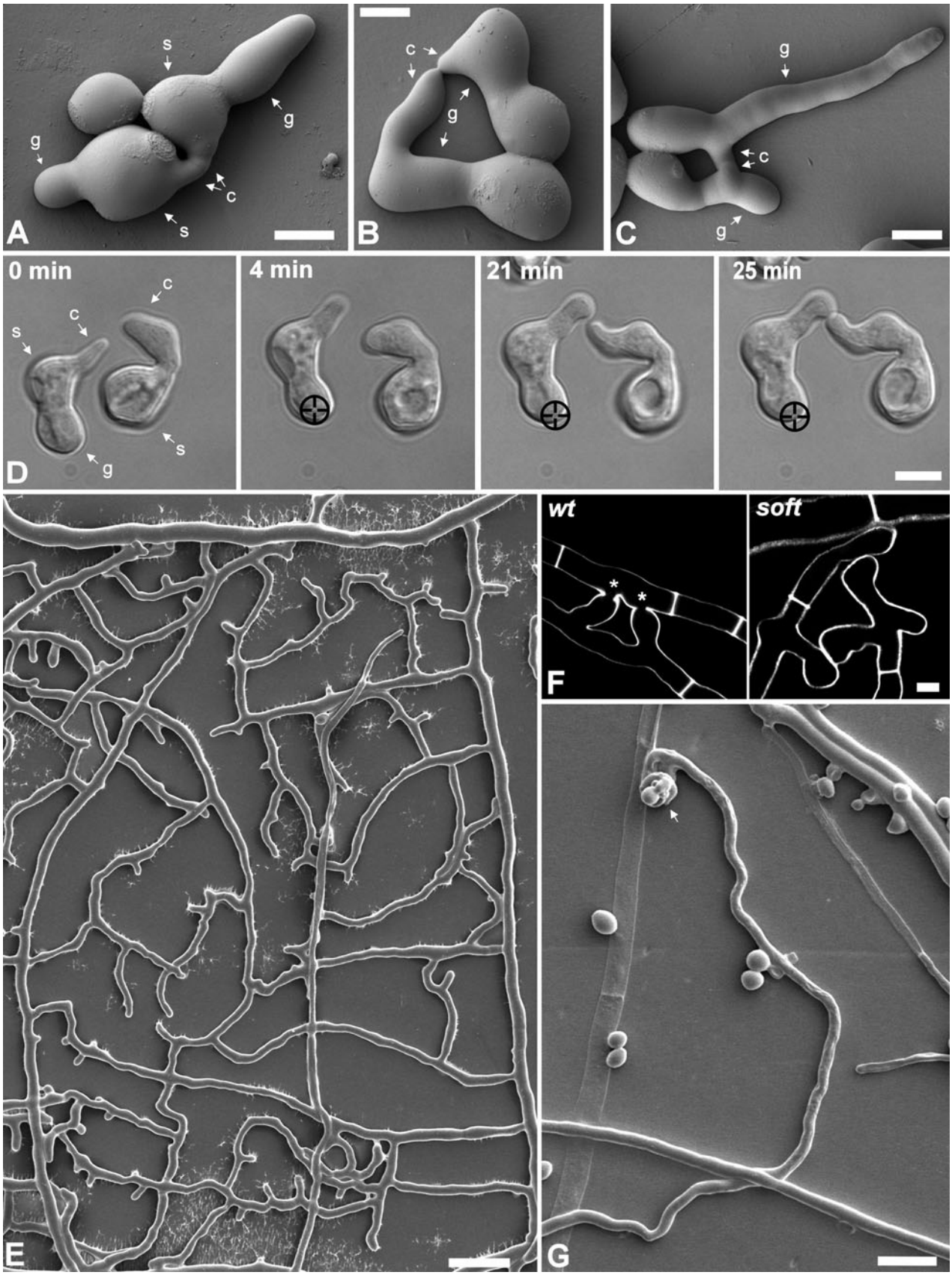
Fusion hypha formation is associated with the development of a Spitzenkörper. The Spitzenkörper is a complex organelle and protein assemblage predominated by secretory vesicles that is found in growing hyphal tips or at sites of branch initiation (Harris et al., 2005; Virag and Harris, 2006), and which is associated with polar and directional growth of hyphae (Bracker et al., 1997; Gierz and Bartnicki-Garcia, 2001; Girbardt, 1957; Grove and Bracker, 1970; Riquelme et al., 1998). During the precontact phase, the fusion hypha Spitzenkörper shares features in common with the Spitzenkörper of a growing vegetative hypha. The secretory vesicles within the Spitzenkörper can be readily stained with the membrane-selective dye FM4-64 (Fischer-Parton et al., 2000; Hickey et al., 2005). By use of this marker dye, the Spitzenkörper was stained in fusion hyphae of *N. crassa* and its behavior was monitored (Hickey et al., 2002). The formation of a new Spitzenkörper was found to precede a new fusion hypha at its site of emergence, and the positive chemotropic reorientation of fusion hyphal tips towards each other was associated with the Spitzenkörper in these tips positioning themselves towards the opposing tips (the localization of the Spitzenkörper in the growing tip was coincident with the direction of homing).

Postcontact Phase

Hyphal fusion in *N. crassa* usually results from "tip-to-tip" contact between two fusion hyphae or from "tip-to-side" contact between a fusion hypha and another hypha (Buller, 1933; Hickey et al., 2002). When the hyphal tips involved in fusion are very short branches, they are often referred to as "pegs" (Buller, 1933). During the postcontact phase, hyphal tips that will fuse cease extension growth and often undergo isotropic growth, which results in swelling of their tips (Hickey et al., 2002). This is accompanied by the adhesion of the two hyphae to each other and the digestion of their intervening cell walls. The Spitzenkörper persists during this period, suggesting that it provides secretory vesicles for wall synthesis during hyphal tip swelling and for the delivery of extracellular adhesives and cell wall-degrading enzymes during cell wall degradation and fusion pore formation (Hickey et al., 2002).

Postfusion Phase

The onset of the postfusion phase is marked by the fusion of the plasma membranes of the two anastomosing hyphae. This results in the formation of a "fusion pore" through which cytoplasm and organelles, including nuclei, flow. Fusion is often associated with dramatic alterations in the bulk flow of cytoplasm and organelles and facilitates their rapid mixing between the fused hyphae. The fusion pore increases in diameter following fusion, and one or two septa commonly form in its vicinity (Hickey et al., 2002). The Spitzenkörper persists at the site of fusion pore formation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and presumably continues to function in providing secretory vesicles containing cell wall-degrading enzymes and proteins associated with fusion pore formation and membrane merger. The Spitzenkörper disappears once the fusion pore is fully formed (Hickey et al., 2002). During the post-contact and postfusion phases, the persistence of the Spitzenkörper within a fusion hypha is a marked behavioral difference compared to that in a vegetative hypha, in which the Spitzenkörper characteristically disappears after hyphal growth has ceased (Girbardt, 1957).

Hyphal Fusion during Fertilization and in Sexual Development

In members of the Ascomycota and Basidiomycota, hyphal fusion occurs during mating-cell fusion and during the formation and maintenance of the dikaryon during the sexual phase of the life cycle. Mating-cell fusion commonly involves the fusion between a compatible hypha and a spore (microconidium or conidium in the Ascomycota and oidium in the Basidiomycota), which acts as a male fertilizing agent (or spermatium) in outbreeding species. In the Ascomycota, a fertile receptive hypha, the trichogyne, is often produced and grows out from the ascogonium of an unfertilized fruiting body. These trichogynes show positive chemotropism towards sex pheromones released by conidia (or microconidia) of opposite mating type (Fig. 1G) (Bistis, 1981; Kim and Borkovich, 2006). Mating cell fusion in some ascomycete species occurs in the absence of conidium-trichogyne fusion, but the precise mechanism(s) involved are poorly understood. In the Basidiomycota, monokaryotic hyphae of basidiomycete species show positive chemotropism towards sex pheromones released by oidia of opposite mating type (Bistis, 1970; Webster, 1980). Alternatively, compatible monokaryotic hyphae of opposite mating type can fuse in the absence of male oidia (Webster, 1980).

The dikaryotic phase is a feature of filamentous ascomycete and basidiomycete species, where nuclei of opposite mating type (in outbreeding species) or two genetically identical nuclei (in inbreeding species) are involved in the formation and proliferation of binucleate cells. In many ascomycete and basidiomycete species, dikaryotic cells are formed and maintained by specialized hyphae called crozier and clamp cells, respectively (Fig. 2A and B).

In *N. crassa* and related ascomycete species, ascogenous hyphae grow out from the ascogonium as a branched hyphal network that is mostly multinucleate (Beckett, 1981; Zickler et al., 1995). A hook-shaped crozier forms at the tip of an ascogenous hypha. Two nuclei within the crozier go through a simultaneous mitotic division and undergo septation across the two mitotic spindles to produce a uninucleate terminal cell and a binucleate penultimate cell, while the fourth nucleus remains in the multinucleate compartment of the ascogenous hypha (Fig. 2A). The two nuclei (the so-called "prefusion nuclei") in the penultimate cell undergo karyogamy. This cell becomes the ascus mother cell and subsequently grows out and develops into the ascus. Meanwhile, the terminal cell grows back and fuses with the subtending ascogenous hypha, allowing the nuclei in these two compartments to pair up. In *N. crassa*, the two nuclei that subtend the ascus also undergo karyogamy (Raju, 1980). In other species, these two nuclei maintain the dikaryotic state; this process is repeated as a result of further croziers forming as branches from the dikaryotic subtending cell (Berteaux-Lecellier et al., 1998; Read and Beckett, 1996). Other patterns of karyogamy have been described in other ascomycete species (e.g., in *Neotiella rutilans*) and have been reviewed by Read and Beckett (1996).

Clamp cells are found at the septa of dikaryotic hyphae of many basidiomycete species. Clamp cell formation is initiated from the apical hyphal compartment and involves the formation of a backward-growing hyphal branch at a site that is just forward of the site of the future septum. The hypha continues to grow back and forms a hook-like structure. Concomitantly, the two genetically different nuclei of the dikaryotic apical hyphal compartment divide and a septum forms at the base of the clamp cell in which one of the nuclei becomes trapped (Fig. 2B). Another septum is formed within the apical hyphal compartment, leaving the nucleus of the other mating type in the newly formed subapical hyphal compartment while keeping together two genetically different nuclei in the apical compartment. A subapical peg is typically formed in response to the presence of the backward-growing clamp cell, and the two fuse, resulting in the two solitary nuclei becoming united in the subapical hyphal compartment. This process is repeated at regular intervals to maintain binucleate hyphal compartments along the dikaryotic vegetative hypha (Badalyan et al., 2004; Buller, 1933; Todd and Aylmore, 1985). Within sexual fruiting bodies, little is known about hyphal fusion between somatic hyphae, although it has been reported in the sexual primordia of various basidiomycete species (Van der Valk and Marchant, 1978; Williams et al., 1985).

FIGURE 1 *N. crassa*. (A) Conidial anastomosis tubes (CATs) (c) that have formed directly from macroconidia (s) and fused with each other. Note that the germ tubes (g) are wider than the CATs. Bar = 5 μm. (From M. G. Roca, C.E. Jeffree, and N. D. Read, unpublished data.) (B) CATs (c) that have formed from germ tube (g) tips, grown towards each other, and made contact. Bar = 5 μm. (From Roca, Jeffree, and Read, unpublished.) (C) CATs (c) that have formed subapically from germ tubes (g) and have fused. Bar = 5 μm. (From Roca et al., 2005b, with permission.) (D) The CAT homing assay. The two conidia had germinated, and their CATs were homing towards each other (0 min). The left-hand germling was repositioned (here shown 4 min after repositioning). The CAT tips then changed their orientation to home back towards each other (15 and 21 min) before making contact (25 min) and subsequently fusing (not shown). The left-hand conidium remained trapped throughout the entire 25-min period without apparent inhibition of CAT growth, homing, or fusion. The position of the trap in the germ tube (g) is represented by the crosshair in the circle. Note that the germ tube is significantly wider than either CAT. Bar = 10 μm. (From Wright et al., 2007, with permission.) (E) Hyphal fusion in a mature colony that has resulted in a complex interconnected hyphal network. Bar = 100 μm. (From K.M. Lord and N. D. Read, cover image for 2008 issues of *Fungal Biology Reviews*, with permission.) (F) A comparison of the morphology of anastomosis between fusion hyphae in a mature wild-type colony (note fusion pores [asterisks]) and a fusion mutant (*soft*) in which fusion does not occur. Hyphae imaged by confocal microscopy after staining with calcofluor white M2R. Bar = 10 μm. (From Fleißner et al., 2005, with permission.) (G) Trichogyne that has homed towards, and wrapped around, a macroconidium (arrow) of opposite mating type. Bar = 20 μm. (From H.C. Kuo, C.E. Jeffree, and N. D. Read, unpublished data).

TABLE 1 Genes required for hyphal fusion in *N. crassa* and their roles in the fusion process

			Required for process?:							
Gene[a]	Locus[a]	Function	Germling fusion	CAT induction	CAT homing	Hyphal fusion in mature colony	Protoperithecium formation	Mating cell fusion	Sexual development[b]	References
NCU09842	*mak-1*	MAPK	?	?	?	Yes	Yes	?	Male fertile	Maerz et al., 2008
NCU06419	*mek-1*	MAPKK	?	?	?	Yes	Yes	?	Male fertile	Maerz et al., 2008
NCU02234	*mik-1*	MAPKKK	?	?	?	Yes	Yes	?	Male fertile	Maerz et al., 2008
NCU02393	*mak-2*	MAPK	Yes	Yes	?	Yes	Yes	?	Ascospore lethal	Pandey et al., 2004; Roca et al., 2005b
NCU04612	*mek-2*	MAPKK	?		?	Yes	Yes	?	Ascospore lethal	Maerz et al., 2008
NCU06182	*nrc-1*	MAPKKK	Yes	Yes	?	Yes	Yes	?	Ascospore lethal	Pandey et al., 2004; Roca et al., 2005b
NCU00340	*pp-1*	TF[c]	Yes	Yes	?	Yes	Yes	?	Ascospore lethal	Li et al., 2005
NCU07024	*os-2*	MAPK	?	?	?	Yes	Yes	?	Male fertile	Jones et al., 2007; Maerz et al., 2008
NCU03071	*os-4*	MAPKKK	?	?	?	Yes	Yes	?	Ascospore lethal	Maerz et al., 2008
NCU00587	*os-5*	MAPKK	?	?	?	Yes	Yes	?	Ascospore lethal	Maerz et al., 2008
NCU09757	*gpig-1*	GPI-anchor	?	?	?	Yes	Yes	?	Lethal	Bowman et al., 2006
NCU06663	*gpip-1*	GPI-anchor	?	?	?	Yes	Yes	?	Lethal	Bowman et al., 2006
NCU07999	*gpip-2*	GPI-anchor	?	?	?	Yes	Yes	?	Male fertile	Bowman et al., 2006
NCU06508	*gpip-3*	GPI-anchor	?	?	?	Yes	Yes	?	Male fertile	Bowman et al., 2006
NCU05644	*gpit-1*	GPI-anchor	?	?	?	Yes	Yes	?	Male fertile	Bowman et al., 2006
NCU02794	*so*	Unknown	Yes	Yes	Yes	Yes	No	No	Perithecia not formed	Fleißner et al., 2005
NCU03727	*ham-2*	Membrane protein	Yes	Yes	?	Yes	No	No	Perithecia not formed	Roca et al., 2005b; Xiang et al., 2002
NCU09337	*Prm-1*	Membrane protein	Yes	No	No	Yes	No	Yes	Ascus dominant	Fleißner et al., 2009

[a]Nomenclature and gene names according to *N. crassa*.

[b]"Male fertile" means that the mutant can be taken through a cross if used as a male, but not as a female. "Ascospore lethal" means that, even if used as a male, ascospore progeny bearing the indicated mutated gene cannot be recovered through a cross. "Perithecia not formed" indicates that mating cell fusion occurs, but subsequent sexual development from the protoperithecium is blocked when the mutant is used as a female. "Ascus dominant" indicates that mating cell fusion occurs, but subsequent sexual development from the protoperithecium is blocked when the mutant is used as a female or a male.

[c]TF, transcription factor.

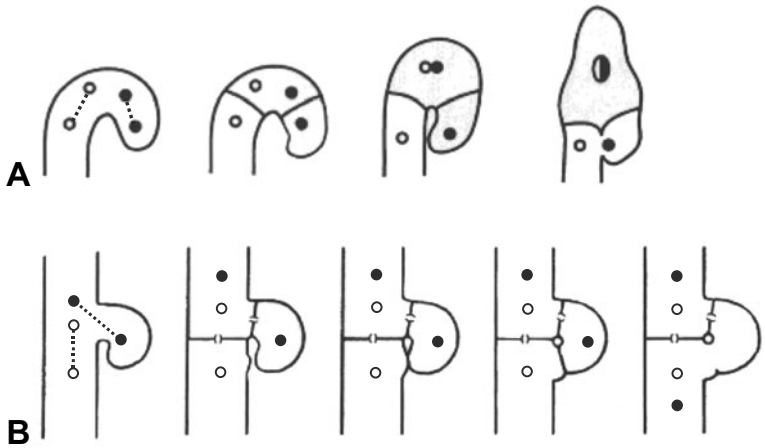

FIGURE 2 (A) Diagram of crozier cell fusion in a typical ascomycete species. (B) Diagram of clamp cell fusion in a basidiomycete species. See "Hyphal Fusion in a Mature Colony" for details.

MECHANISTIC BASIS OF VEGETATIVE HYPHAL FUSION

Induction

CAT induction probably involves an extracellular CAT inducer produced by ungerminated conidia. Evidence for this comes from observations that CAT formation is dependent on macroconidial concentration (Roca et al., 2005b), CATs form when conidia are close together, and culture filtrates can induce CAT fusion (Leu, 1967). CAT induction may involve a form of quorum sensing (i.e., a mechanism whereby cells sense their density by detecting extracellular molecules secreted by the population) (Miller and Bassler, 2001). In *Venturia inaequalis*, germling fusion via CATs was found to be frequent when conidia were in close proximity. Fusion was not observed between two isolated conidia mounted close to one another (Leu, 1967). However, when multiple conidia were placed close to one another under the same conditions, numerous germling fusion events were observed. Fusion between isolated conidial pairs in *V. inaequalis* was induced by the addition of culture filtrates from the same or different isolates. The observed fusion induction was apparently species specific, because culture filtrates of *Venturia pirina* did not induce fusions between *V. inaequalis* conidia. The identity of the self-signaling inducer is unknown.

The composition of the growth medium can affect fusion frequency among conidial germlings, and this may relate to the frequency of CAT induction. In *Leptosphaeria coniothyrium*, *Sclerotinia fructigena*, *Botrytis* sp., *Fusarium* sp., and *N. crassa*, the frequency of germling fusion was reduced on rich organic media (such as 1% malt or potato dextrose agar), but fusion was frequent when these media were diluted (Köhler, 1930; Laibach, 1928). In contrast, the frequency of germling fusion in *V. inaequalis* was found to be significantly greater on complete medium than on basal medium or water agar (Leu, 1967). It is not clear whether a nutrient or another factor in the medium is causing these inhibitory or stimulatory effects.

CAT induction in *N. crassa* requires a putative transmembrane protein (HAM-2) (Xiang et al., 2002) and the NRC-1/MEK-2/MAK-2 mitogen-activated protein (MAP) kinase pathway (see below); strains containing mutations in the genes encoding these proteins do not form CATs (Roca et al., 2005b). Furthermore, phosphorylation of MAK-2 increased during the period when CAT formation/hyphal fusion is most prolific (Pandey et al., 2004). In *Saccharomyces cerevisiae*, stimulation of the pheromone response pathway required for mating cell fusion results in activation of a transcription factor, Ste12 (Errede and Ammerer, 1989), which regulates the expression of genes involved in polarized growth, cell adhesion, cell wall breakdown, and membrane merger (Cross, 1988; Gammie et al., 1998; Heiman and Walter, 2000) (see "Chemoattraction" below). In *N. crassa*, a strain containing a deletion of the *ste12* ortholog, *pp-1*, is very similar in phenotype to the *nrc-1*, *mek-2*, and *mak-2* mutants, fails to form CATs, and is defective in germling fusion and hyphal fusion in mature colonies (Li et al., 2005; J. Li, A. Fleißner, and N. L. Glass, unpublished observations) (Table 1). CAT induction may also involve a filamentous-ascomycete-specific WW domain protein called SOFT (SO) because *so* mutants produce fewer CATs than the wild type (Fleißner et al., 2005).

Much less is known about what makes hyphae in the subperipheral region of the mature colony competent to undergo fusion, but the process involves the induction of specialized fusion hyphae (see "Hyphal Fusion in a Mature Colony" above). It is not known whether this is primarily regulated by a program of gene expression that is specific to this region of the colony or whether environmental factors are important in initiating the expression of hyphal fusion genes. However, an unknown self-signaling inducer is involved. This inducing compound seems to be produced by the tips of fusion hyphae because the close proximity of a fusion hyphal tip to a trunk hypha in *N. crassa* can induce the formation of a new fusion hypha in its vicinity (Buller, 1933; Hickey et al., 2002). It is not unreasonable to postulate that the same molecule may function as both the CAT and fusion hypha inducer.

As indicated in "Hyphal Fusion in a Mature Colony" above, nematode traps produced by nematophagous fungi result from hyphal fusion. They can be induced by the presence of nematodes, and Pramer and Stoll (1959) coined the term "nemin" to describe the inducer. Nordbring-Hertz et al. (1989) later showed that di- and tripeptides containing valine were very effective in inducing traps under nutrient-poor conditions.

Chemoattraction

A key feature of CATs or fusion hyphae is that they are attracted towards one other. In *N. crassa*, when the relative

position of two conidial germlings showing mutual attraction is changed by micromanipulation using optical tweezers, the CAT tips of both germlings adjust their growth towards each other to make contact and undergo fusion (Fleißner et al., 2005; Roca et al., 2005b; Wright et al., 2007) (Fig. 1D). These observations provide compelling evidence for a diffusible chemoattractant released from CAT tips and for a chemoattractant receptor located at the CAT tips. However, as with the CAT/fusion hypha inducer(s) (see "Induction" above), the identity of the chemoattractant is unknown. It is possible that the inducer and chemoattractant are the same self-signaling ligand. In *S. cerevisiae*, both the induction of the formation of mating cell protuberances (shmoos) and chemotropic interactions associated with mating are regulated by the secretion of peptide sex pheromones (**a**-pheromone or α-pheromone) that bind to their cognate G-protein-coupled receptors (Ste3p or Ste2p, respectively) in a cell of the opposite mating type (Cross, 1988; Elion, 2000).

The process of vegetative hyphal fusion between compatible cells in filamentous fungi involves the fusion of genetically identical cells (i.e., self fusion), in contrast to mating-cell fusion in *S. cerevisiae*, which involves the fusion of two genetically nonidentical cells (i.e., nonself fusion). The best-studied example of chemoattraction between genetically identical cells in eukaryotes is found in the slime mold *Dictyostelium discoideum*, in which extracellular cyclic AMP (cAMP) acts as a chemoattractant that results in aggregation of amoebae (Manahan et al., 2004). The discovery that the *N. crassa* genome encodes three G-protein-coupled receptor-like proteins that resemble *Dictyostelium* cAMP receptors (Borkovich et al., 2004; Galagan et al., 2003) suggested the possibility that cAMP may serve a similar chemoattractant role in filamentous fungi. However, cAMP plays no apparent role as a CAT chemoattractant in *N. crassa*, because a *cr-1* mutant lacking cAMP was shown to undergo CAT homing after optical tweezer-based micromanipulation (Roca et al., 2005b).

In *S. cerevisiae*, the interaction of **a** or α-pheromone with Ste3p or Ste2p, respectively, results in the disassociation of a heterotrimeric G-protein (Gα=Gpa1p, Gβ=Ste4p, and Gγ=Ste18p). Interaction between Gβγ and Ste20p activates a MAP kinase cascade (Ste11p-Ste7p-Fus3p) (Cross, 1988; Elion, 2000). Although mating-cell fusion in *N. crassa* involves a similar interaction of cognate pheromones and G-protein-coupled receptors (Li et al., 2007), mutations in *N. crassa* genes encoding the sex pheromones (*mfa-1* and *ccg-4*), the pheromone receptors (*pre-1* and *pre-2*), and heterotrimeric G-proteins (*gna-1*, *gnb-1*, and *gng-1*) do not affect vegetative hyphal fusion, as assessed by heterokaryon tests and germling fusion assays (Kim and Borkovich, 2004, 2006; Li et al., 2007). However, strains containing deletion mutations in *mak-2* (the *N. crassa* ortholog of *FUS3*) and *nrc-1* (the *STE11* ortholog) are unable to undergo germling or hyphal fusion in mature colonies (Pandey et al., 2004; Roca et al., 2005b) (Table 1). Furthermore, conidia of *mak-2* and *nrc-1* mutants do not attract wild-type CATs, indicating that neither of these mutants produces chemoattractant (Table 1) (Roca et al., 2005b). We have recently found that MAK-2-green fluorescent protein (GFP) localizes to the tips of CATs undergoing chemotropic homing towards each other (A. Fleißner, M. G. Roca, N. D. Read, and N. L. Glass, unpublished observations). These studies indicate that the MAK-2 MAPK pathway is required both for the initiation of fusion and for chemotropic interactions. Recent data indicate that a strain containing a deletion mutation of the predicted MAPKK in the MAK-2 MAP kinase pathway, termed *mek-1*, is also vegetative-fusion defective (Maerz et al., 2008) (Table 1). Interestingly, the vegetative-fusion defects of *mak-2* and *nrc-1* mutants are suppressed by mutations in *cot-1* (Maerz et al., 2008); *cot-1* encodes a protein kinase of the NDR Ser/Thr protein kinase family, which is required for hyphal tip elongation in *N. crassa* (Yarden et al., 1992). The requirement of the MAK-2 MAP kinase pathway for vegetative hyphal fusion is also conserved in other filamentous ascomycete fungi. For example, in *A. nidulans*, a mutant disrupted in the MAPKKK *nrc-1* homolog, *ste*C, fails to form heterokaryons (Wei et al., 2003).

In addition to genes encoding components of the MAK-2 MAP kinase pathway, an *N. crassa* locus called *soft,* which encodes a filamentous ascomycete-specific WW domain protein, is required for vegetative hyphal fusion (Fig. 1F) (Fleißner et al., 2005; Wilson and Dempsey, 1999). *so* mutants form CATs, but these fail to show chemotropic interactions (Table 1; Fig. 3), suggesting that *so* mutants are impaired both in the production/secretion and/or reception/transduction of a chemoattractant signal (Fleißner et al., 2005). Functional SO-GFP is found in the cytoplasm in hyphae, but upon injury, SO-GFP localizes rapidly to septal pore plugs. SO-GFP localization was shown to be Woronin body (*hex-1*) independent. Importantly, *so* mutants show a decrease in efficiency of septal plugging upon injury (Fleißner and Glass, 2007). In the homothallic filamentous ascomycete species *Sordaria macrospora*, strains containing mutations in the *so* ortholog (*pro40*) are also defective in vegetative hyphal fusion (Rech et al., 2007); Pro40, like SO, localizes to septal pore plugs (Engh et al., 2007). Similarly, mutations in *so* orthologs in plant pathogens such as *Fusarium oxysporum* and *Alternaria brassicicola* also resulted in mutants that fail to undergo vegetative hyphal fusion (Craven et al., 2008; Prados Rosales and Di Pietro, 2008) (see "Hyphal Fusion and Plant Pathogenesis" below).

In *N. crassa,* the *ham-2* (acronym for hyphal anastomosis) locus encodes a putative transmembrane protein (Xiang et al., 2002); *ham-2* mutants fail to undergo both germling and hyphal fusion in mature colonies (Table 1; Fig. 3). Similar to the *mak-2* and *nrc-1* mutants described above, *ham-2* mutants fail to attract wild-type CATs, suggesting that HAM-2 is required for chemoattractant production/secretion and/or reception/transduction (Roca et al., 2005b). In *S. cerevisiae*, mutations in the *ham-2* ortholog, *FAR11*, result in mutants that prematurely recover from G_1 growth arrest following exposure to pheromone (Kemp and Sprague, 2003). Far11p was shown to interact with five other proteins (Far3p, Far7p, Far8p, Far9p, and Far10p). Homologs of genes encoding *FAR3* and *FAR7* are lacking in *N. crassa* (Glass et al., 2004). Preliminary data show that mutations in *N. crassa* homologs of *Far*8 and *Far9/10* (*ham-3* and *ham-4*, respectively) result in mutants displaying phenotypes similar to the *ham-2* mutant, including a block in vegetative hyphal fusion (A. Simonin, C. G. Rasmussen, M. Yang, and N. L. Glass, unpublished results). In *S. macrospora*, strains containing mutations in the *ham-2* ortholog (*pro22*) results in mutants that show a dramatic reduction in fusion frequency and fail to develop mature fruiting bodies (Rech et al., 2007). The predicted *N. crassa* HAM-3 protein shows significant similarity to proteins of the striatin family. The striatin family of proteins act as scaffolding proteins that organize signaling complexes; for example, formation of a complex between striatin and the

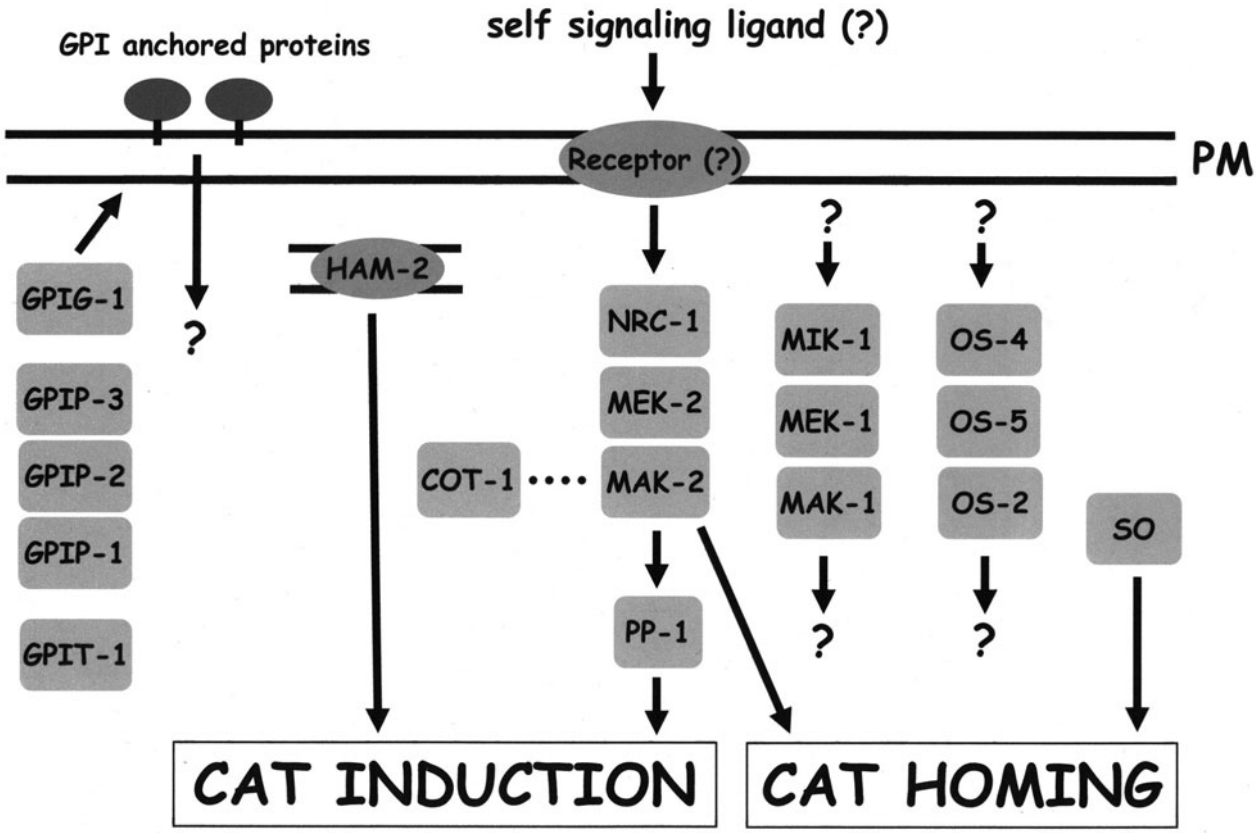

FIGURE 3 Working model of the signaling involved during vegetative hyphal fusion in *N. crassa* (see the text for details). The self-signaling ligand and receptor responsible for the process of self-fusion are unknown. GPIG1, GPIT1, GPIP1, GPIP2, and GPIP3 are involved in the GPI protein anchoring pathway (Bowman et al., 2006). It is not known which stage(s) of vegetative hyphal fusion these proteins regulate. HAM-2 is a predicted transmembrane protein (Xiang et al., 2002), although it is not known which cellular membrane it is associated with. NRC1-MEK2-MAK2 (Li et al., 2005; Maerz et al., 2008; Pandey et al., 2004; Roca et al., 2005b), MIK1-MEK1-MAK1 (Maerz et al., 2008), and the OS4-OS5-OS2 (Maerz et al., 2008) are three MAP kinase pathways, and PP1 (Li et al., 2005) is the transcription factor predicted to be at the base of the MAK2 pathway. Upstream elements of the MAK1 and OS2 MAP kinase pathways and the stage(s) of vegetative hyphal fusion that they regulate are unknown. Mutations in *cot-1* suppress the vegetative hyphal fusion defect of *mak-2* (Maerz et al., 2008). SO is an ascomycete-specific WW domain protein (Fleißner and Glass, 2007; Fleißner et al., 2005).

estrogen receptor is required for estrogen-induced activation of a MAP kinase signal transduction pathway (Lu et al., 2004). In *S. macrospora*, mutations in the *ham-3* ortholog (*pro11*) result in a mutant unable to complete sexual development; full fertility was restored by expression of a mouse striatin cDNA (Poggeler and Kuck, 2004). These data suggest that HAM-2/Pro22, HAM-3/Pro11, and HAM-4 might physically interact in filamentous ascomycete fungi and that this interaction might be involved in vegetative hyphal fusion.

In the tips of hyphae within the mature colony, including chemotropic fusion hyphae, the position of the Spitzenkörper determines directional growth (Harris et al., 2005; Hickey et al., 2002; Virag and Harris, 2006). However, an obvious Spitzenkörper is not observed in germ tubes (Araujo-Palomares et al., 2007) or CATs (Roca et al., 2005b). The absence of a recognizable Spitzenkörper may be due to the reduced concentration of vesicles in the slow-growing germ tube and CAT tips, and this may relate to their slow extension rates. Nevertheless, germ tube and CAT tips probably possess key components of the hyphal tip growth machinery such as the polarisome and exocyst protein complexes that have been characterized in *S. cerevisiae*. The polarisome is responsible for directing cytoskeletal and other cell components towards sites of localized cell expansion at sites of growth, while the exocyst is involved in secretory vesicle docking and fusion with the plasma membrane (Harris et al., 2005; Virag and Harris, 2006). Chemotropic growth must involve the rapid transduction of chemoattractant signals perceived at the plasma membrane to the tip growth machinery (Spitzenkörper/polarisome/exocyst). This machinery responds rapidly and sensitively to direct secretory vesicles involved in wall synthesis to appropriate locations on the apical plasma membrane of CATs or fusion hyphae (Read, 2007). This is thought to involve the directed polymerization of actin microfilaments, along which secretory vesicles are transported towards sites of vesicle fusion within the tips of CATs and fusion hyphae.

Of possible significance here is that cytochalasin-A and latrunculin B, drugs that disrupt F-actin polymerization, perturb germling fusion (Rasmussen, 2007; M. G. Roca and N. D. Read, unpublished data).

Cell-Cell Adhesion, Cell Wall Breakdown, and Membrane Merger

The molecular basis of cell-cell adhesion, a shift from polar to isotropic growth, cell wall degradation, and plasma membrane merger during the final stages of hyphal fusion are little understood in filamentous fungi. Upon physical contact, the two Spitzenkörper of two fusion hyphae are juxtaposed at the point of contact (Hickey et al., 2002). The localization of the two Spitzenkörper in fusion hyphae resembles the prefusion complexes found during muscle myoblast fusion, in which vesicles line up at the sites of cell-cell contact, forming pairs across the apposing plasma membranes (Dworak and Sink, 2002).

In *S. cerevisiae*, mating-type-specific adhesins are expressed in response to pheromone, enabling the fusion partners to tightly adhere at their shmoo tips (reviewed by Lipke and Kurjan, 1992). However, *S. cerevisiae* genes encoding mating-type-specific agglutinins are not conserved in the genome of *N. crassa* (Glass et al., 2004), indicating that different proteins may be involved in cell-cell adhesion. Recent data from *N. crassa* demonstrate that glycosylphosphatidylinositol (GPI)-anchored proteins are required for hyphal fusion. Mutations in *gpig-1*, *gpip-1*, *gpip-2*, *gpip-3*, and *gpit-1* genes, which encode components of the *N. crassa* GPI anchor biosynthetic pathway, resulted in mutants with pleiotropic phenotypes and that were unable to undergo vegetative hyphal fusion, as assessed by heterokaryon formation (Bowman et al., 2006) (Table 1; Fig. 3). Chitinases, such as the GPI-anchored chitinase ChiA in *A. nidulans*, which localizes to the tips of germ tubes, at hyphal branching sites, and at hyphal tips (Yamazaki et al., 2008), might play a role in cell wall remodeling and degradation at sites of hyphal fusion during fusion pore formation.

In *S. cerevisiae*, one of the few proteins implicated in plasma membrane merger during mating is Prm1p; *prm1*Δ mutants show a ~50% reduction in mating cell fusion (Heiman and Walter, 2000). Deletions of the *PRM1* homolog in *N. crassa* (Δ*Prm-1;* mutations in *Prm-1* are ascus dominant) showed a ~50% reduction in germling fusion (Fleißner et al., 2009). In addition, *N. crassa* Δ*Prm-1* mutants have a similar reduction in the frequency of trichogyne-conidium fusion during sexual reproduction, as well as defects in crozier cell fusion (see "Relationship between Vegetative Hyphal Fusion and Sexual Development" below). These data suggest that in *N. crassa*, PRM1 is involved in cell fusion events during both the vegetative and sexual stages of the *N. crassa* life cycle and may thus be part of the general cell fusion machinery.

Other Proteins Involved in Vegetative Hyphal Fusion

A number of mutants have been found to be defective in vegetative hyphal fusion, but the precise stages at which they are blocked have yet to be determined (Table 1). These include strains containing mutations in two additional MAP kinase pathways predicted in filamentous ascomycete genomes (Lengeler et al., 2000; Xu, 2000). The first of these MAP kinase pathways is orthologous to the *S. cerevisiae* cell wall integrity MAP kinase pathway and includes a MAPKKK (Bck1p), two redundant MAPKKs (Mkk1p/2p), and a MAPK (Slt2p/Mpk1p). In *S. cerevisiae*, the polarized growth of mating projections involves new cell wall synthesis, which requires activation of a cell wall integrity MAP kinase, Mpk1p (Slt2p) (Buehrer and Errede, 1997); during pheromone-induced morphogenesis, Mpk1-GFP is localized at the shmoo tip (Changwei et al., 2007). In *F. graminearum*, mutations in the *MPK1* ortholog, *MGV1*, resulted in a mutant that fails to form heterokaryons via hyphal fusion (Hou et al., 2002). Similarly, in *N. crassa*, mutations in the *MPK1* ortholog, *mak-1*, and predicted upstream kinases, *mek-1* and *mik-1*, also result in strains that fail to undergo hyphal fusion (Maerz et al., 2008) (Table 1; Fig. 3). In *M. grisea* and *Colletotrichum lagenarium*, mutations in the MAP kinase gene orthologous to *MPK1* affect conidial germination, sporulation, ability to form appressoria, and plant infection (Kojima et al., 2002; Xu, 2000; Xu et al., 1998). Defects in vegetative fusion were not assessed in these mutants.

A third MAP kinase pathway predicted in filamentous ascomycete genomes is orthologous to the osmosensing MAP kinase pathway in *S. cerevisiae* and has been extensively studied in a number of filamentous ascomycete fungi (Furukawa et al., 2005; Vitalini et al., 2007; Zhang et al., 2002). In *N. crassa*, strains containing deletion mutations in the MAPKKK (*os-4*), the MAPKK (*os-5*), or the MAPK (*os-2*) genes show hyphal fusion defects (Maerz et al., 2008) (Table 1). Future studies will reveal the relationship, interaction, and function of these MAPK pathways in vegetative hyphal fusion (Fig. 3).

RELATIONSHIP BETWEEN FUSION DURING VEGETATIVE GROWTH AND SEXUAL DEVELOPMENT

Relationship between CAT Fusion and Hyphal Fusion in a Mature Colony

CAT fusion is being used as a model to study fundamental aspects of vegetative hyphal fusion because it is a much simpler and more experimentally amenable system than fusion within a mature colony (Read and Roca, 2006). Data obtained so far suggest that many features of CAT fusion are common to the fusion of hyphae in a mature colony. All germling fusion mutants identified so far are also defective in hyphal fusion in mature colonies (Fleißner et al., 2005; Roca et al., 2005b). However, there are differences between the two processes of hyphal fusion. First, there are morphological and developmental differences. CATs are short (Fleißner et al., 2005; Roca et al., 2005b), while fusion hyphae vary from being short peg-like structures to much longer, and often dichotomously branched hyphae (Hickey et al., 2002). Second, there are physiological differences. Cytoplasmic and organelle mixing between hyphae is usually very rapid between hyphae in the mature colony following fusion but is very slow between fused CATs (Roca and Read, unpublished). We speculate that there might be slight differences in the turgor pressures of fusion hyphae which result in the rapid bulk flow of cytoplasm and organelles (Hickey et al., 2002). It may be that the turgor pressure differential between fusing germlings is much less or nonexistent (Read and Roca, 2006). Further identification and characterization of hyphal fusion mutants should more clearly define similarities and differences between fusion of CATs and fusion hyphae and should determine how far CAT fusion can be used as a model for vegetative hyphal fusion in general.

Relationship between Vegetative Hyphal Fusion and Sexual Development

All of the fusion mutants so far identified in *N. crassa* have a pleiotropic phenotype and are affected in aspects of sexual development (Table 1). For example, the *N. crassa mak-2*, *mek-2*, and *nrc-1* mutants fail to form female reproductive structures (protoperithecia) or develop defective ones, suggesting that hyphal fusion may be important during the development of fruiting bodies (e.g., in the development of the protoperithecial or perithecial wall). Fusion mutants also show reduced growth rates and conidiation defects (Kothe and Free, 1998; Li et al., 2005; Maerz et al., 2008; Pandey et al., 2004; A. Lichius, K. M. Lord, and N. D. Read, unpublished results). Similar results have been observed with hyphal fusion mutants identified in other species (Craven et al., 2008; Hou et al., 2002; Rech et al., 2007; Vallim et al., 2000; Wei et al., 2003).

Two stages of sexual development in *N. crassa* and related species that involve hyphal fusion are mating-cell fusion and crozier cell fusion (see "Hyphal Fusion in a Mature Colony" above). Mechanistically mating cell fusion appears reminiscent of germling and hyphal fusion in mature colonies: cells communicate over a spatial distance, grow towards each other, and fuse. However, the molecular basis of vegetative and sexual cell communication may differ. In particular, pheromone, pheromone receptor, and G-protein mutants are defective in chemotropic interactions during mating but are apparently not defective in vegetative fusion (see "Chemoattraction" above). In contrast, mutations in *soft* (*so*) result in strains that lack chemotropic interactions between CATs and are defective in germling fusion and fusion between hyphae in the mature colony (Fleißner et al., 2005). Both the *N. crassa so* mutant and the *S. macrospora so* mutant (*pro40*) form protoperithecia (Engh et al., 2007), but further sexual development is blocked. However, in *N. crassa*, the *so* mutant shows normal trichogyne-conidium chemotropic interactions and undergoes normal mating-cell fusion (Table 1). In addition, fusion of crozier cells in fruiting bodies (perithecia) of both the *so* and the *ham-2* mutants are not affected (Fleißner et al., 2005; Xiang et al., 2002). These data support the hypothesis that cell-cell communication during vegetative hyphal fusion and fusion during sexual development employ different signaling molecules and respective receptors for chemotropic interactions. In contrast, the *N. crassa* Δ*Prm-1* mutants have normal chemotropic interactions during germling and mating cell fusion but show ~50% reduction in both germling and trichogyne-conidium fusion (Fleißner et al., 2009). These observations indicate that vegetative and sexual fusion may require common components of the machinery involved in plasma membrane merger. Future comparison of different types of hyphal fusion at different stages during the fungal life cycle will be important to distinguish molecular components universally involved in cell fusion from those that are specific to individual cell fusion pathways.

ROLES OF HYPHAL FUSION

Cooperation between Conidial Germlings

Cooperation among relatives is a ubiquitous phenomenon in ecological systems and involves the activities of one individual benefiting one or more other individuals (for a review, see Sachs et al., 2004). Germling fusion during colony establishment is an excellent example of cooperative behavior; cooperation within and between fungal individuals can affect fitness attributes of filamentous fungi (Pringle and Taylor, 2002). Most germling and hyphal fusion mutants show pleiotropic growth defects, indicating a role for many of these genes in processes in addition to vegetative hyphal fusion. Thus, effects of germling fusion on cooperation and colony development are difficult to assess. However, one fusion mutant, *so*, shows a wild-type maximal linear colony extension rate (Fleißner et al., 2005), although *so* mutants show a significant delay in achieving wild-type rates. These observations suggest that cooperation via germling fusion during colony establishment may result in the attainment of a maximal extension rate by the mature colony in a shorter period of time (Roca and Read, unpublished).

Maintenance of Physiological Homeostasis in a Mature Colony

Hyphal fusion results in a supracellular, syncytial state in a filamentous fungal colony. This interconnected organization enables translocation of cellular contents, such as organelles, metabolites, nutrients, or signaling compounds throughout the colony, presumably facilitating growth and reproduction (Buller, 1933; Rayner, 1996). In *N. crassa*, fusion is often associated with dramatic alterations in cytoplasmic flow; organelles, including nuclei, pass through fusion pores (Hickey et al., 2002). Such dramatic changes in cytoplasmic flow and movement of organelles suggest that filamentous fungi must adapt to the physiological consequences of hyphal fusion within a fungal colony. Anastomosis between hyphae within a single colony allows the cooperation of hyphae and enables fungi to establish complex functional units that show coordinated growth and exploration of their environment (Buller, 1933; Rayner, 1996). Cytoplasmic continuity can be restored by growth of hyphae through dead hyphal compartments, followed by hyphal fusion with living sectors (Buller, 1933). Self fusion between multiple colonies can allow them to act cooperatively in supporting one or more large fruiting bodies, such as toadstools (Buller, 1933). Thus, in general terms, vegetative hyphal fusion contributes significantly to the general homeostasis within a colony.

Heterokaryon Formation and Parasexuality

Anastomosis between hyphae of genetically different, but heterokaryon-compatible genotypes, can lead to genetic diversity via parasexual recombination and formation of novel genotypes (Pontecorvo, 1956; Swart et al., 2001). This process has been postulated to contribute to the high adaptability of fungi in species that lack sexual reproduction and genetic diversity generated via meiotic recombination. However, in nature, the formation of heterokaryons is restricted by heterokaryon incompatibility (also termed vegetative or somatic incompatibility) (see chapter 20; see also Glass and Dementhon, 2006; Leslie, 1993; Saupe, 2000; and Worrall, 1997). Thus, at least for filamentous ascomycete species, it is unclear how much gene flow and recombination occur within natural fungal populations as a consequence of hyphal fusion and heterokaryon formation.

Hyphal Fusion and Plant Pathogenesis

Hyphal fusion can also be important in the pathogenicity of some plant pathogens. Studies involving a number of plant pathogenic fungi have shown that genes encoding components of MAP kinases are essential for infection of host plants (Kojima et al., 2002; Lev et al., 1999; Takano et al.,

2000; Xu, 2000; Xu et al., 1998). These observations led to the hypothesis that cell fusion and plant infection might share common signaling pathways and that cell fusion might be a prerequisite for successful host colonization (Craven et al., 2008; Prados Rosales and Di Pietro, 2008). For example, mutation of *mak-2* MAPK orthologs in plant pathogenic fungi such as *M. grisea*, *C. lagenarium*, or *Cochliobolus heterostrophus* results in strains that are defective in appressorium formation and also fail to colonize host plants when inoculated through wound sites (Lev et al., 1999; Takano et al., 2000; Xu, 2000; Xu et al., 1998). In the non-appressorium-forming "ergot fungus" *Claviceps purpurea*, mutations in the *mak-2* ortholog similarly result in nonpathogenic strains that show an inability to infect through preformed lesions (Mey et al., 2002). For wild-type *C. purpurea*, strictly oriented growth along the pollen tube path to reach the base of the ovary is a prerequisite of successful host infection (Tudzynski and Scheffer, 2004). Mutations in the MAP kinase gene orthologous to *N. crassa mak-1* in *M. grisea* and *C. lagenarium* result in strains affected in conidial germination, sporulation, ability to form appressoria, and plant infection (Kojima et al., 2002; Xu, 2000; Xu et al., 1998); defects in vegetative fusion have not been determined in these mutants. It is unclear whether defects associated with pathogenesis in these mutants are a consequence of a lack of vegetative hyphal fusion or whether they relate to alternative roles of MAP kinase pathways in these fungal pathogens.

Recent studies of the interaction between *F. oxysporum* and tomato revealed that conidia germinating on the root surface readily fused with each other, forming a mycelial network that adhered to the plant surface. Strains containing mutations in genes encoding orthologs of *mak-2* or *so* (*fmk1* or *fso1*, respectively) were germling fusion defective. Interestingly, mycelia of the *fmk1* and *fso1* mutants were easily detached from the root surface, indicating a function for network formation in plant colonization. However, while the *fmk1* mutant was unable to infect the plant, *fso1* strains showed only slightly reduced virulence (Prados Rosales and Di Pietro, 2008), suggesting that germling fusion might contribute to infection but is not essential for pathogenicity. In contrast, inactivation of the *so* ortholog in *A. brassicicola* (*Aso1*) led to a mutant exhibiting hyphal fusion defects as well as a lack of pathogenicity on the host plant (cabbage). While the mutant was able to penetrate and initially colonize the host, it failed to invade plant tissue. It was suggested that hyphal anastomosis in *A. brassicicola* may play a role in facilitating transport of nutrients from the host plant throughout the invading colony (Craven et al., 2008). The extent to which anastomosis contributes to virulence and pathogenicity might very well depend on the individual infection strategies of different phytopathogenic species. Future analyses employing a variety of different host pathogen systems will be essential to fully reveal the roles of hyphal fusion during fungal pathogenesis.

COMPARISON OF HYPHAL FUSION WITH OTHER SYSTEMS INVOLVING CELL FUSION

Many of the processes required for hyphal fusion in filamentous fungi during vegetative growth are also required during cell fusion processes in general, including signaling by diffusible substances, directed cell growth or movement towards each other, attachment of the two cell types to one another, production and targeting of enzymes to the attachment site, and fusion of the plasma membranes of the interacting cells. Hyphal fusion in filamentous fungi is comparable to somatic cell aggregation and fusion events in other eukaryotic organisms. In cellular slime molds such as *D. discoideum*, amoebae exhibit a form of self signaling and show chemoattractive movement towards each other (Manahan et al., 2004). The adjustment of hyphal growth towards the fusion partner is comparable to cell polarization and shmoo formation during yeast mating (Kurjan, 1993), directed pollen tube growth towards the ovary (Higashiyama et al., 2003), or the extension and/or stabilization of filopodia during myoblast fusion (Chen and Olson, 2004). Cell fusion is involved in the fusion of multinucleate vegetative plasmodia of the acellular slime mold *Physarum polycephalum* resulting in the formation of larger plasmodia (Collins and Haskins, 1972). In colonial marine invertebrates, such as *Hydractinia* and *Botryllus*, fusion can occur between compatible colonies (Buss and Grosberg, 1990; Litman, 2006), while in mammals, examples of somatic cell fusion events that result in syncytia include myoblast fusion during muscle differentiation, fusion between osteoclasts in bone formation, and trophoblast fusion during placental development (Chen et al., 2007; Cross et al., 1994; Dworak and Sink, 2002; Jee and Nolan, 1963; Paululat et al., 1999; Shemer and Podbilewicz, 2003; Vignery, 2000). Understanding the molecular basis of hyphal fusion during vegetative growth in filamentous fungi may provide a paradigm for self-signaling and self-fusion mechanisms in eukaryotic microbial species, as well as provide a useful model for somatic cell fusion events in complex, multicellular species.

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Biology and Genetics of Vegetative Incompatibility in Fungi

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DEFINITION OF VEGETATIVE INCOMPATIBILITY

As an individual fungal colony grows in nature, it will interact with a range of organisms inhabiting the same niche, including other individuals belonging to the same species. Filamentous fungi have the ability to undergo somatic cell fusion. As a consequence, an encounter between two individuals belonging to the same species can lead to spontaneous heterokaryon formation. In most cases, however, when somatic cell fusion occurs between distinct natural isolates of a given species, the fusion cell is adversely affected to various extents. The adverse reaction ranges from a simple growth impairment to an acute cell death reaction. This phenomenon is known as vegetative (or heterokaryon) incompatibility (VI). VI can be envisioned as a conspecific somatic self/nonself recognition process analogous to other somatic allorecognition processes described in other phyla. Fusion between genetically distinct individuals is known to exist in protists, plants, and animals (Buss, 1982), but it is particularly common in fungi because they readily form hyphal anastomoses. Therefore, it is perhaps not surprising that allorecognition is so widespread among filamentous fungi. In Basidiomycetes, VI is typically expressed between secondary mycelia (i.e., stable-mating-type heterokaryons) but appears to be overridden in sexually compatible homokaryons (Rayner, 1991). In Ascomycetes, somatic incompatibility is expressed directly between homokaryotic mycelia, which generally outcross nonetheless, since somatic incompatibility is often suppressed during the sexual cycle.

As is detailed below, the VI reaction is triggered by genetic differences between fungal individuals and is defined by precise gene-to-gene interactions. Incompatibility is determined by the interaction of specific alleles at polymorphic loci termed heterokaryon incompatibility (*het*) genes. The *het* genes have been best studied in the Ascomycetes *Podospora anserina* and *Neurospora crassa* (Glass and Dementhon, 2006; Saupe, 2000). *het* genes control the cellular coexistence of genetically dissimilar nuclei by determining, on the basis of allelic or nonallelic interactions, whether cell death will occur. Stable heterokaryons form only when the strains are compatible at all *het* loci.

From a general point of view, VI can be viewed as an example of genome conflict. Different nuclear components lead to an adverse reaction when present in the same cell in a heterokaryon. As such, VI bears resemblance with phenomena like hybrid lethality or sterility (Burke and Arnold, 2001), hybrid necrosis in plants (Bomblies et al., 2007; Bomblies and Weigel, 2007), and speciation based on gene-to-gene interactions (Presgraves, 2007). Similar to VI, in all these phenomena the heteroallelism at polymorphic loci (in a heterokaryon, a zygote, or a meiotic progeny) cannot be tolerated and leads to detrimental cellular effects.

Fungi can have a very long life span (Smith et al., 1992) and may encounter many potentially dangerous viruses, bacteria, mites, insects, and nematodes, as well as other fungi of the same or other species. The study of VI has so far mainly focused on conspecific interactions, and VI has been considered to be a potential defense mechanism against disadvantages associated with somatic cell parasitism and cytoplasmic infections. However, a recent study suggests that the VI reaction may also be relevant during interactions with microbial parasites. The plant pathogen *Pseudomonas syringae* may have acquired a *het* gene as a potential virulence factor to trigger the VI reaction in *N. crassa* and utilize the fungus as a sole nutrient source (Wichmann et al., 2008). Interspecies host-pathogen interactions may be relevant for understanding the biological role, evolution, and maintenance of polymorphisms in VI genes in filamentous fungi (see below). This view is of particular relevance when one considers that other examples of genome conflicts are apparently a secondary consequence of gene divergence imposed by host-pathogen interactions (Bomblies and Weigel, 2007).

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ASSAYS FOR VI

Vegetative compatibility between conspecific strains has been tested using different methods. When antagonistic alleles are expressed in the same cell (when introduced by anastomosis, transformation, protoplast fusion, or sexual crossing), the direct manifestations of VI can be studied at the macroscopic (barrage or abnormal growth) or microscopic (cell death) level. Alternatively, the differential consequences of anastomosis between compatible and incompatible pairings for stable heterokaryon formation (heterokaryon complementation of recessive markers) or heteroplasmon (e.g., exchange of suppressive mitochondrion-based senescence) can be used. It must be noted that some of the following methods are used more frequently than others.

Barrage Formation or Cell Death of Fused Cells

A barrage can often be seen on agar medium when incompatible colonies are confronted with one another (Fig. 1). The reactions may vary from dark lines between the colonies to a clear zone with lethal hyphal fusions. The barrage assay is convenient because it does not require any specific genetic marker and can be performed directly using wild-type isolates. It is thus very commonly used for directly analyzing natural populations and has been utilized in population studies of fungi, for example, *Ophiostoma ulmi* (Brasier, 1983), *Cryphonectria parasitica* (Anagnostakis, 1987), *P. anserina* (Bernet, 1967), and *Phomopsis subordinaria* (Meijer et al., 1994). It must be noted that the barrage reaction is often dependent on the experimental conditions. With *P. anserina*, for example, a clear barrage zone is formed on dung media whereas a white opaque zone is observed on synthetic media. With *N. crassa*, barrage formation occurs only on low-nutrient media (Micali and Smith, 2003). The absence of macroscopic antagonistic reactions is therefore not always a reliable indication of vegetative compatibility (Micali and Smith, 2003). In Basidiomycetes, demarcation lines are often observed between paired mycelia on agar media and on wood in nature (Worrall, 1997).

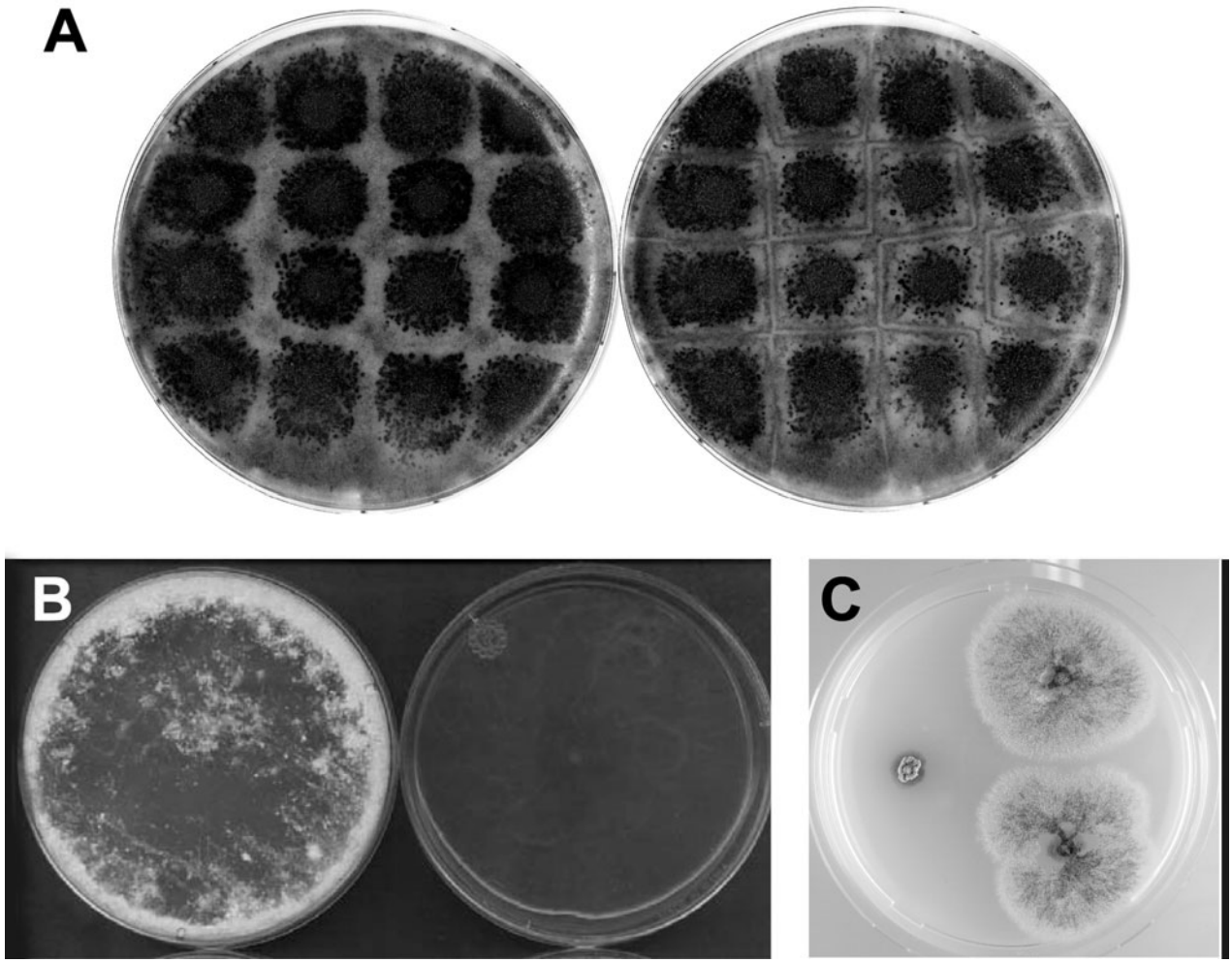

FIGURE 1 Incompatibility assays, barrage tests, and forced heterokaryon tests. (A) Barrage test in *P. anserina*. Strains on the left plate are compatible and show normal contact zones; the strains on the right plate are incompatible and show a barrage reaction, which on this medium appears as a clear zone lined by pigmented lines. (B) Forced heterokaryon test in *N. crassa*. The left plate contains compatible forced heterokaryon; the right plate *het-c/pin-c* shows incompatible heterokaryon. (C) Forced heterokaryon assay in *Aspergillus*. The inoculate on the left corresponds to an incompatible heterokaryon that fails to establish; the colonies on the right correspond to compatible heterokaryons with different spore color markers.

Microscopic observations of VI triggered by hyphal fusion between incompatible strains have revealed similar effects in different species: hyphal compartmentation, vacuolization, and cell death of the fused cells and often also of the neighboring cells (Aimi et al., 2002a, 2002b; Anagnostakis, 1984; Pinan-Lucarre et al., 2007). In *Rhizoctonia solani,* VI has indeed been based on lysis of anastomosed cells (Carling et al., 1994; Julian et al., 1996); lysis of the anastomosing and adjacent cells is seen when the anastomosing isolates are vegetatively incompatible.

Heterokaryon Formation

Two types of heterokaryons have been reported in filamentous ascomycete fungi. In the first type, heterokaryons are limited only to actual fusion cells and nuclei do not migrate between cells (Puhalla and Mayfield, 1974). Heterokaryons are continually re-formed by repeated fusion events within the mycelium and are visualized as vigorous growth in the contact zone. In the second type, in species such as *N. crassa* and *P. anserina*, heterokaryotic cells proliferate and almost all cells within the mycelium are heterokaryotic (Perkins, 1988).

Conidia from different auxotrophic mutant strains of *N. crassa* but from the same genetic background typically show complementation and grow readily on minimal medium (Beadle and Coonradt, 1944). However, when mutant strains from different genetic backgrounds are used, such complementation often does not occur, indicative of VI (Fig. 1). If the strains are incompatible, the forced heterokaryon fails to establish or displays growth retardation and aberrant morphology and shows evidence of cell death when examined microscopically. This principle of complementation of recessive marker alleles in a heterokaryon has been used in various ascomycete species and requires the introduction of genetic markers. For such studies, chlorate-resistant, nitrate-nonutilizing markers have been widely used. Such mutants can be isolated on the basis of chlorate resistance and classified for their ability to grow on various N sources (Cove, 1976). Nitrate-nonutilizing mutants have been used in heterokaryon tests to assay for compatibility groups among naturally occurring isolates in a number of different fungi. Alternatively, wild-type strains can be directly screened against tester strains that carry both a recessive auxotrophic and a dominant resistance marker, as has been demonstrated for *Aspergillus niger* (van Diepeningen et al., 1997). In *Aspergillus nidulans*, complementation of color mutations in asexual spores has been used as evidence for heterokaryosis (Grindle, 1963).

Introduction of Antagonistic *het* Alleles or Products into the Same Cell or Nucleus

Assays introducing antagonistic *het a*lleles or products into the same cell or nucleus have been performed in various ways and with different consequences. Cell death may occur when incompatible cytoplasm is directly introduced into the cell, e.g., by microinjection in *N. crassa* (Garnjobst and Wilson, 1956) or by maternal inheritance of the HET-s prion in *P. anserina* (Dalstra et al., 2005). More generally, VI is manifested as abnormal growth, morphology, and pigmentation after introduction of antagonistic *het* alleles, e.g., by transformation (Micali and Smith, 2006; Saupe et al., 1996a, 1996b) or protoplast formation (Dales and Croft, 1990). In pseudohomothallic species, such as *P. anserina* and *Neurospora tetrasperma*, outcrossing may result in incompatible combinations of *het* alleles in the sexual progeny leading to self-incompatible progeny, abortion, or loss of the heterokaryotic state of ascospores (Bernet, 1967; Pinan-Lucarre et al., 2007; Saenz et al., 2001). In *N. crassa*, loci involved in VI have been assessed by using strains containing chromosomal translocations (Perkins, 1997). When such strains are crossed to wild-type isolates, one-fourth of the progeny contain partial duplications for the translocated segment. If progeny have alternate *het* specificity in the duplicated segment, partial diploid progeny will show a typical VI phenotype (Mylyk, 1975; Perkins, 1988). Translocation strains have been useful in assessing specificity at single *het* loci in wild-type isolates of *N. crassa* (Wu et al., 1998). Similar to forced heterokaryons, ultrastructural studies of incompatible partial diploids in *N. crassa* show organelle degeneration, shrinkage of the plasma membrane, and septal plugging (Jacobson et al., 1998).

Transfer of Senescence

In several fungi, the transfer of cytoplasmic elements is not completely blocked by VI. However, there is a significant delay in the spread of highly suppressive lethal conditions, such as vegetative death in *A. nidulans* (Caten, 1972), mitochondrially based senescence in *P. anserina* (Marcou, 1961), and plasmid-based senescence in *Neurospora* (Debets et al., 1994). In compatible, but not in incompatible pairings of isolates, a mixed culture has the life span of the senescent strain. A VI test based on this principle has been used to assay field isolates of *Neurospora intermedia* (Debets et al., 1994).

The VI results of the barrage and heterokaryon assays do not systematically overlap. The relationship between barrage formation and VI shows concordance in *P. anserina* (Bernet, 1965). However, in *N. crassa, C. parasitica, Sclerotinia sclerotiorum, Rosellinia necatrix*, and *Tuber borchii* (Aimi et al., 2002a, 2002b; Ford et al., 1995; Micali and Smith, 2003; Sbrana et al., 2007; Smith et al., 2006), barrage formation was not necessarily associated with genetic differences at *het* loci. For instance, in *C. parasitica,* heteroallelism at one of the incompatibility loci (*vic4*) is detected in barrage assays but not in forced heterokaryon assays (Smith et al., 2006). In *N. crassa*, a barrage reaction occurs between strains identical at all major *het* loci, while barrages are not observed between strains with genetic differences at *het-6, het-c,* and *mat* (Micali and Smith, 2003). These observations suggest that, in some cases, barrage formation functions independently of VI.

Classification of Fungal Populations into vcgs

Incompatibility assays can be used to classify individuals in a fungal population into vegetative compatibility groups (vcgs). The number of different vcgs in populations can be considerable. One recent study on vcg diversity in native populations of the chestnut blight fungus, *C. parasitica*, indicated that in Japan and China almost every isolate belonged to a unique vcg (Liu and Milgroom, 2007). Another study, involving *Fusarium oxysporum*, identified 96 different vcgs in a sample of 128 isolates (Alves-Santos et al., 1999). In fungi that lack a sexual stage, have infrequent sexual recombination, or primarily inbreed, it is often inferred that strains belonging to the same vcg represent clonal derivatives from a common ancestor. Analysis of vcgs in populations is often used by plant pathologists to characterize the structure and dynamics of fungal populations.

GENETICS OF HETEROKARYON INCOMPATIBILITY

Two main types of genetic determinism of VI can be distinguished. In allelic systems, VI is determined by the interaction of two incompatible alleles of the same locus. In

nonallelic incompatibility, the VI reaction is caused by the interaction of specific alleles from distinct loci. The total number of *het* loci varies from species to species. In Ascomycetes it is usually around 10 (Anwar et al., 1993; Cortesi and Milgroom, 1998; Jinks et al., 1966; Perkins and Davis, 2000; Pinan-Lucarre et al., 2007). In Basidiomycetes, the number of VI loci is smaller, from one to four (Kauserud et al., 2006; Lind et al., 2007; Marcais et al., 2000; Worrall, 1997). For allelic systems, the number of allelic variants at a given locus is generally low (two or three alleles). The allelic systems are apparently the more common type of incompatibility systems. However, as is shown in the following section, the boundaries of this classification are somewhat blurred by the fact that more complex situations are also encountered; for instance, there are allelic systems in which the two allelic forms have totally different sequences or systems in which closely linked genes display nonallelic interactions that genetically appear allelic.

Genes involved in VI have been identified so far in only two species: *N. crassa* and *P. anserina*. Two categories of genes involved in VI have been characterized. The first category includes *het* genes that encode recognition function and are polymorphic between individuals. The second category includes downstream or upstream effector genes, in which mutations suppress or attenuate phenotypes associated with VI. The different incompatibility systems that have been characterized at the molecular level in *N. crassa* and *P. anserina* are shown in Fig. 2.

N. crassa

The Mating-Type Locus and the *tol* Suppressor

In *N. crassa*, the *mat A-1* and *mat a-1* mating-type genes are essential to define mating identity and for sexual reproduction. However, *mat A-1* and *mat a-1* function as a *het* locus during somatic cell fusions (Glass et al., 1988); heterokaryons between *A* and *a* strains are severely inhibited in their growth and aconidial and show cell death (Beadle and Coonradt, 1944; Garnjobst and Wilson, 1956; Pittenger, 1957). In other words, the result of the interaction between the *mat A-1* and *mat a-1* genes is completely different, depending on the cellular context in which it takes places.

The *mat a* and *mat A* mating-type alleles occupy the same locus in *a* and *A* strains, respectively, but are completely different in DNA and amino acid sequence; they are termed idiomorphs (Glass et al., 1988, 1990). The *mat A* idiomorph bears three different genes: *mat A-1*, *mat A-2*, and *mat A-3*. *mat A-1* specifies mating identity and is also required for postfertilization functions, while *mat A-2* and *mat A-3* operate only during postfertilization events (Ferreira et al., 1998, 1996). The *mat a* idiomorph contains a single gene, *mat a-1*, which is responsible for all mating and postfertilization

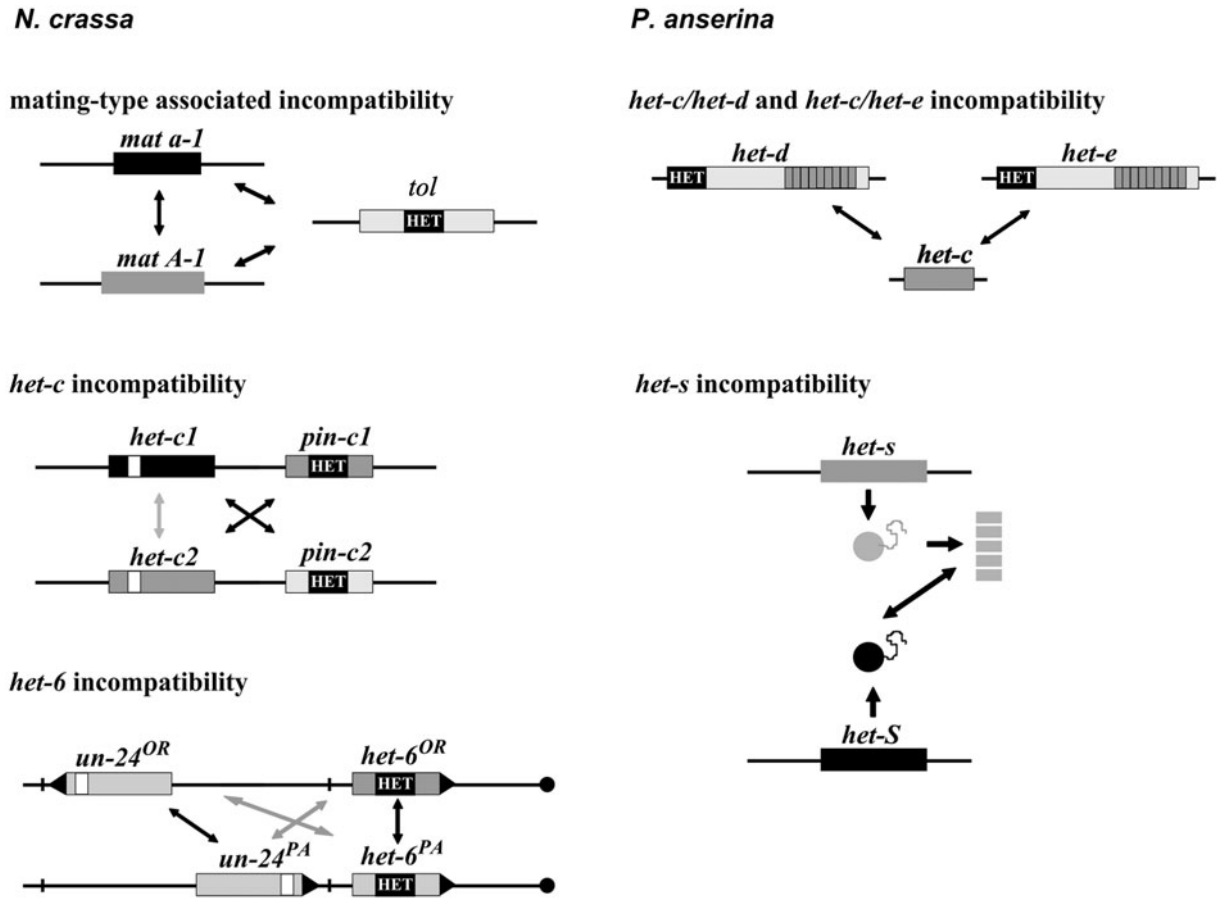

FIGURE 2 The different incompatibility systems characterized at the molecular level in *N. crassa* and *P. anserina*. The black double arrows represent incompatible interactions. The gray double arrows represent interactions that contribute to the VI response. The HET domain is represented by a black box. The specificity regions in *het-C* and *un-24* are represented by a white box. See the text for details.

functions (Staben and Yanofsky, 1990). The *mat a-1* and *mat A-1* gene pair confers the incompatibility function (Glass et al., 1990; Staben and Yanofsky, 1990). The MAT a-1 and MAT A-1 polypeptides are transcription factors displaying an HMG and an α-box, respectively. The mating and incompatibility functions are genetically separable in MAT a-1 and MAT A-1. Mutations or deletions that specifically affect only one of the functions have been described (Saupe et al., 1996a, and references therein). This observation led to the suggestion that MAT a-1 and MAT A-1 specify mating and somatic identity by distinct mechanisms.

This hypothesis is further supported by the fact that mutations at the unlinked *tol* locus suppress mating-type-associated incompatibility but do not affect mating capacity (Newmeyer, 1970) (Fig. 2). The *tol* gene encodes a 1,011-amino-acid polypeptide displaying a leucine-rich repeat (LRR) motif and a HET domain (Shiu and Glass, 1999). LRRs are protein-protein interaction domains consisting of repeated sequences. LRRs are encountered in a large number of proteins that are often involved in recognition functions, such as gene families associated with pathogen recognition in plants (DeYoung and Innes, 2006). As discussed below, the HET domain is a fungus-specific protein domain found in a variety of proteins involved in VI. *tol* is expressed in vegetative tissue but not in developing fruiting bodies, which could explain why the *mat a-1-mat A-1* interaction only leads to VI and cell death during the vegetative stage (Shiu and Glass, 1999).

The *het-c/pin-c* Locus

The *het-c/pin-c* system involves two adjacent genes located on LGII (Kaneko et al., 2006; Saupe and Glass, 1997; Saupe et al., 1996b). *het-c* encodes a plasma membrane protein with a large glycine-rich unstructured domain (Sarkar et al., 2002; Saupe et al., 1996b). Three distinct allelic types have been described for *het-c* (Saupe and Glass, 1997). The alleles differ in a highly polymorphic region of 34 to 48 amino acids. This region determines allelic specificity (Saupe and Glass, 1997; Wu and Glass, 2001). During the incompatibility reaction, a heterocomplex between alternate incompatibility polypeptides forms at the plasma membrane (Sarkar et al., 2002).

The adjacent gene *pin-c* (named for "partner for incompatibility with *het-c*") encodes a protein with a HET domain (Kaneko et al., 2006). Incompatibility between alternate *het-c* alleles can be suppressed by mutations in *pin-c*. Three different *pin-c* alleles have been described. The level of polymorphism between the *pin-c* alleles is extreme, ranging from 62 to 76% nucleotide identity, depending on the gene pair considered. *het-c/pin-c* gene pairs form three distinct haplotypes. The incompatibility function of these haplotypes is determined by three additive interactions: two nonallelic *het-c-pin-c* interactions and an additional allelic *het-c* interaction that contributes to the severity of the VI phenotype (Fig. 2) (Kaneko et al., 2006). Thus, this VI system, which at first glance is genetically an allelic system, is in fact based primarily on nonallelic interactions.

A screen for suppressors of *het-c/pin-c* incompatibility identified a gene called *vib-1* (for "vegetative incompatibility blocked"), which encodes a putative transcription factor showing homology to *Saccharomyces cerevisiae* Ndt80 (Dementhon et al., 2006; Xiang and Glass, 2002). Mutations in *vib-1* also suppress mating-type-associated incompatibility and incompatibility determined by the *het-6/un-24* system (Xiang and Glass, 2004). The suppressive effect is due to the fact that *vib-1* positively regulates expression of *pin-c*, *tol*, and *het-6* HET domain encoding genes. Expression of these genes is barely detectable in a Δ*vib-1* strain (Dementhon et al., 2006).

The *het-6/un-24* System

The third incompatibility system of *N. crassa* that has been characterized at the molecular level is the *het-6/un-24* system (Micali and Smith, 2006; Mir-Rashed et al., 2000; Smith et al., 2000a, 2000b). The *het-6* locus bears two tightly linked genes designated *het-6* and *un-24*, each having two allelic variants (Oakridge/OR and Panama/PA) (Fig. 2). In natural populations, only *het-6*^{OR} *un-24*^{OR} and *het-6*^{PA} *un-24*^{PA} allele combinations have been found; these allele combinations define the OR and PA haplotypes, respectively. The *het-6* gene encodes a protein with a HET domain, and *un-24* encodes the large subunit of ribonucleotide reductase, an essential enzyme of DNA metabolism. The sequence divergence between *het-6* alleles is considerable: the *het-6*^{OR} and the *het-6*^{PA} gene products share only 68% amino acid identity (Micali and Smith, 2006). In contrast, polymorphism in the *un-24* gene product is limited to a region located at the C terminus of the ribonucleotide reductase. The OR and PA haplotypes differ in overall genomic organization; the divergence between the haplotypes has been generated by a 18.6-kbp paracentric inversion (Micali and Smith, 2006). The existence of this inversion provides an explanation for severe linkage disequilibrium observed between *het-6* and *un-24*.

P. anserina

The het-c/het-d and *het-c/het-e* Systems

The *het-c*, *het-d*, and *het-e* genes of *P. anserina* define two nonallelic systems (*het-c/het-d* and *het-c/het-e*) (Bernet, 1965; Pinan-Lucarre et al., 2007). All three loci are multiallelic. The *het-c/het-d* and *het-c/het-e* incompatibilities also lead to sexual incompatibility, a situation not encountered for the *N. crassa* VI systems (Bernet, 1965). The *het-c* gene encodes a glycolipid transfer protein (GLTP) conserved in fungi, plants, and animals (Brown and Mattjus, 2007; Mattjus et al., 2003; Saupe et al., 1994). (Note that in spite of the confusing homonymy, *P. anserina het-c* and *N. crassa het-c* are not homologous.) *P. anserina het-c* is homologous to ACD11, a GLTP involved in programmed cell death (PCD) in higher plants (Brodersen et al., 2002). Het-c is required for proper ascospore delimitation and maturation (Saupe et al., 1994). *Het-d* and *het-e* are paralogues and encode large multidomain proteins (Espagne et al., 2002; Saupe et al., 1995). Both comprise an N-terminal HET domain followed by a central NACHT domain and a C-terminal WD-repeat domain. NACHT domains are regulatory NTP-binding domains involved in oligomerization and found in a variety of proteins controlling PCD and pathogen recognition processes (Leipe et al., 2004). NACHT proteins are part of a larger family, termed STAND, which includes other regulators of PCD, such as mammalian APAF-1 (Leipe et al., 2004). WD-repeats adopt a circular β-propeller fold forming a protein-protein interaction platform (Smith et al., 1999). *het-d* and *het-e* are in fact part of a large gene/pseudogene family in *P. anserina* that includes 10 members that share the NACHT and WD-repeat domains (Paoletti et al., 2007). Polymorphism in the *het-d* and *het-e* alleles lies in the WD-repeat domain, specifically at positions located at the protein-protein interaction surface (Espagne et al., 2002; Paoletti et al., 2007). Polymorphism in the HET-C GLTP localizes at the protein surface. It is probable that HET-C/HET-D and HET-C/HET-E incompatibility involves direct interaction of the

GLTP with the polymorphic positions of the WD-repeat of HET-D and HET-E.

Nonallelic incompatibility in *P. anserina* can be suppressed by mutations in the *mod-A* and *mod-B* genes (Barreau et al., 1998). Mutations in *mod-A* lead to partial suppression, while complete suppression of the *het-c/het-d* and *het-c/het-e* VI phenotype occurs only when mutations in *mod-A* and *mod-B* are associated.

The *het-s* System

The *het-s* allelic system is the only incompatibility system described so far for which no involvement of a HET domain has been detected. The *het-s* locus exists as two alleles, designated *het-s* and *het-S* (Rizet, 1952). HET-s and HET-S are proteins of 289 amino acids that differ at 13 amino acid positions distributed over the entire sequence, rather than being clustered in a discrete polymorphic region (Deleu et al., 1993; Turcq et al., 1991). Strains of the *het-s* genotype can exist as two alternate phenotypes, designated [Het-s*] and [Het-s] (Beisson-Schecroun, 1962; Rizet, 1952; reviewed by Saupe, 2007). [Het-s*] strains are compatible with [Het-S] strains, while [Het-s] strains are incompatible with [Het-S] strains. The [Het-s*] and [Het-s] phenotypes are metastable: [Het-s*] strains can acquire the [Het-s] phenotype spontaneously at a low frequency when subcultured. The [Het-s] phenotype can in turn be lost when mycelia are regenerated from propagules that contain very little cytoplasm. For instance, a fraction (~1%) of the mycelia that regenerate after plating of protoplasts from a [Het-s] strain have the [Het-s*] phenotype (Malato et al., 2007). The [Het-s] phenotype is contagious and cytoplasmically transmitted from a [Het-s] to a [Het-s*] strain. In other words, when a [Het-s*] strain is confronted with a [Het-s] strain, it is systematically converted to the [Het-s] phenotype. The [Het-s] phenotype then invades the mycelium at a high rate, reaching several millimeters per hour (Beisson-Schecroun, 1962).

The epigenetic properties and cytoplasmic inheritance of [Het-s] are explained by the fact that the *het-s*-encoded protein is a prion (Coustou et al., 1997). Prions are self-propagating protein particles responsible for fatal neurodegenerative diseases in mammals, but prions also exist in fungi, where they are detected as cytoplasmically transmitted, non-Mendelian genetic elements (Benkemoun and Saupe, 2006). The HET-s protein has two conformational states: a soluble monomeric form in [Het-s*] strains and an infectious aggregated form in [Het-s] strains (Coustou-Linares et al., 2001). In vitro studies showed that the aggregated prion form of HET-s corresponds to an amyloid polymer (Dos Reis et al., 2002; Maddelein et al., 2002; Ritter et al., 2005). Amyloids are fibrillar protein aggregates that display a so-called cross-β structure. HET-s is a two-domain protein with an N-terminal globular domain (spanning amino acid residues 1 through 220) and a C-terminal domain responsible for prion propagation and amyloid formation (Balguerie et al., 2003). This prion-forming domain (PFD) (residues 218 to 289) is unstructured in the soluble form of the protein and adopts a cross-β amyloid fold in the aggregated form (Balguerie et al., 2003). In spite of the tremendous technical difficulties associated with the structural characterization of amyloids, the structure of the HET-s PFD in the prion form was solved recently by solid-state nuclear magnetic resonance, making it the first prion protein to be characterized at that level of resolution (Wasmer et al., 2008). The HET-s amyloids display an exceptionally high level of order not generally found in other amyloids. The HET-s(218–289) displays a pseudorepeat structure with two layers of β-strand per monomer. The fold displays a triangular hydrophobic core leading to proper segregation of the polar residues and charged residues at the fibril surface, while apolar side chains are not exposed to the cellular milieu. This highly organized fold might provide an explanation for the fact that HET-s amyloid fibrils do not display any intrinsic toxicity in vivo.

HET-s and HET-S proteins differ both in the globular domain and in the region corresponding to the HET-s PFD (Balguerie et al., 2003; Turcq et al., 1991). Mutational analyses and chimeric gene constructions have revealed that polymorphisms relevant for specifying the incompatibility type actually reside in the globular domain (Balguerie et al., 2003; Deleu et al., 1993). Single point mutations in the globular domain of HET-S are sufficient to switch incompatibility specificity from [Het-S] to [Het-s]. In HET-S, both the globular domain and the region corresponding to the HET-s PFD are necessary for the incompatibility function. In the case of HET-s, the PFD is required for the incompatibility function, but large deletions in the globular domain do not affect incompatibility function (Balguerie et al., 2004). The mechanism underlying the incompatibility reaction in the *het-s* system remains unknown, in spite of the extensive structural characterization of the HET-s protein. A model compatible with the current data is that during the anastomosis of [Het-s] and [Het-S] strains, HET-s amyloid seeds trigger the conversion of HET-S to an amyloid form. In this model, in contrast to the HET-s amyloids, the HET-S amyloids would display an intrinsic toxicity that is dependent on the HET-S globular domain.

The *het-s/het-S* antagonism is also expressed during the sexual cycle (Bernet, 1965). *het-s* is a spore killer locus (Dalstra et al., 2003, 2005). In a *het-s* female × *het-S* male cross performed at 18°C, in a fraction of the asci, the two ascospores of *het-S* genotype abort, leading to an excess in *het-s* progeny over the expected Mendelian ratio. The *het-s* allele thus behaves as a meiotic drive element when the HET-s protein is in the prion state in the female parent. The *het-s* system provides an example of a genome conflict that can lead to different outcomes depending on the cellular and developmental context in which the antagonistic interaction occurs.

The molecular characterization of the *het*-genes summarized here has revealed that, in contrast to the situation suggested by formal genetic analyses, at the molecular level, VI nonallelic interactions (in the sense of interaction of distinct proteins rather than polymorphic variants of the same protein) are the rule, rather than the exception (Kaneko et al., 2006).

The HET Domain and the Modular Concept of Incompatibility

Five of the six incompatibility systems characterized so far include a gene bearing a HET domain (*tol* in mating-type incompatibility, *pin-c* in *het-c* incompatibility, *het-6* in the *un-24 het-6* haplotype, and *het-d* and *het-e* in the *het-c/d/e* system) (Fig. 2). The only exception to date is the *het-s* prion system, which apparently relies on a very specific toxicity mechanism related to the amyloid-forming ability of the involved proteins. No other VI system displays *het-s*-like epigenetic characteristics, again suggesting that *het-s* is an exception.

The HET domain spans about 200 amino acids and is defined by three conserved blocks of 15 to 30 amino acids with invariant tryptophan residues in each block (Smith et al., 2000b) (Pfam entry, P06985). Constitutive overexpression of the isolated HET domain of the HET-E protein leads to induction of a cell death reaction that is cytologically related

to the VI cell death response (Paoletti and Clave, 2007). Importantly, this cell death reaction is independent of *het-c* (the normal partner of *het-e* in VI) and can be suppressed as the *bona fide het-c/het-e* VI response by mutations in the *mod-A* and *mod-B* genes. These data are consistent with a modular conception of VI, with one protein module involved in the recognition step per se and a second protein module responsible for triggering the VI-associated cell death response. In the *het-c/het-e* system, the recognition module would be constituted by the polymorphic WD-repeat domain and the HET-C protein, while the HET domain of HET-E would correspond to the cell death execution domain. It has been proposed that the transduction of the recognition step into activation of the HET death domain might be based on a mechanism related to the one found in other STAND proteins, like the mammalian APAF-1 apoptosis-inducing factor (Paoletti and Clave, 2007). In APAF-1, the CARD death domain interacts with the WD-repeats until binding of cytochrome *c* to the WD-repeats leads to exposure of the CARD death domain.

The HET domain is found only in members of the Pezizomycota, and all genomes from sequenced Pezizomycota species possess at least one protein with a HET domain (Fig. 3). Fifty-five HET domain proteins are found in the *N. crassa* genome, while 129 are found in *P. anserina*. This number of HET domain genes far exceeds the number of *het* loci that have been characterized genetically in these species. It is thus very unlikely that all these HET domain proteins are involved in VI. It has been proposed that HET domain genes have undergone massive expansion in members of the Pezizomycota to serve different functions in recognition or host defense mechanism, whose exact nature remains unknown (Fedorova et al., 2005). Although Basidiomycetes have VI systems (Worrall, 1997), no HET domain genes have been identified in any basidiomycete genome sequenced so far. This observation suggests that Basidiomycetes have devised an alternative evolutionary solution to the problem of somatic nonself recognition.

CELL BIOLOGY OF VEGETATIVE INCOMPATIBILITY

Compatible Fusions

Hyphal fusion is a ubiquitous phenomenon in filamentous fungi (Glass and Fleißner, 2006; Glass et al., 2004; see also chapter 19), including filamentous basidiomycete, ascomycete, and zygomycete species (Buller, 1933; Gregory, 1984; Rayner, 1996; Giovannetti et al., 1999, 2001; Griffin and Perrin, 1960). Similar to cell fusion in other organisms, the process of hyphal fusion in filamentous fungi requires cell recognition, adhesion, cell wall breakdown, and membrane merger. In *N. crassa*, hyphae at the periphery of the colony exhibit negative autotropism and are refractory to fusion (Trinci, 1984). Within the interior of a colony, hyphae often show positive chemotropic responses (homing) associated with hyphal fusion events (Buller, 1933; Hickey et al., 2002). Both hyphal tip growth and the formation of lateral branches are associated with a membrane-dense organelle, the Spitzenkörper (Girbardt, 1957; Hickey et al., 2002; Lopez-Franco and Bracker, 1996; Riquelme and Bartnicki-Garcia, 2004; Riquelme et al., 1998). During hyphal fusion, the Spitzenkörper of the fusion hyphae are always juxtaposed (Hickey et al., 2002). Phenotypic aspects of compatible hyphal fusion are similar among filamentous fungi, including *T. borchii* (Sbrana et al., 2007), *C. parasitica* (Newhouse and MacDonald, 1991), and the basidiomycetes *Phanerochaete velutina* and *Stereum hirsutum* (Ainsworth and Rayner, 1986). Chemotropic interactions and rare fusion events between different fungal species have also been reported (Köhler, 1930).

Incompatible Fusions

If two fungal colonies of the same species undergo hyphal fusion but have genetic differences at *het* loci, most frequently, the heterokaryotic fusion cell is compartmentalized and dies or is inhibited in its growth. The triggering of hyphal compartmentation and death following hyphal

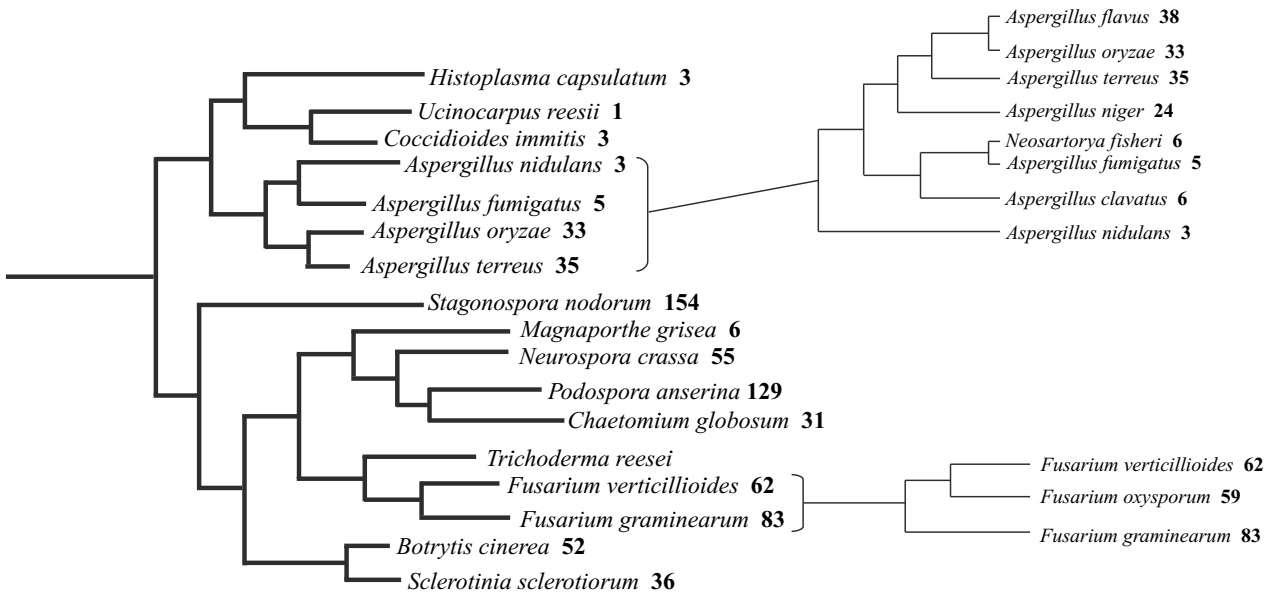

FIGURE 3 Distribution of HET domain proteins in the genomes of sequenced Pezizomycota species. For each species, the number of HET domain proteins in the genome is given on the right. The phylogenetic tree was modified from Fitzpatrick et al., 2006, to include the additional species that have been more recently sequenced.

fusion between *het*-incompatible individuals is morphologically similar among different fungi (Aimi et al., 2002a, 2002b; Ainsworth and Rayner, 1986; Beisson-Schecroun, 1962; Biella et al., 2002; Garnjobst and Wilson, 1956; Newhouse and MacDonald, 1991; Rizet, 1952). Common features include septal plugging, vacuolization of the cytoplasm, organelle degradation, and shrinkage of the plasma membrane from the cell wall. Microscopic characteristics of cell death associated with incompatible fusions are similar to VI in forced heterokaryons, partial diploids, and transformants (Glass and Dementhon, 2006; Perkins, 1988). The frequency of cell death in forced heterokaryons and partial diploid progeny is characteristic of a particular *het* interaction in *N. crassa*, with approximately 30% of the hyphal compartment dying in *het-c/pin-c* interactions, while a smaller percentage of cell death was observed in incompatible *mat* heterokaryons (Xiang and Glass, 2002, 2004).

One of the earliest events associated with an incompatible fusion event is the rapid plugging of septa in the heterokaryotic fusion cell and often in surrounding cells. Presumably, septal plugging prevents transfer of deleterious cytoplasmic elements following the fusion event. In filamentous ascomycete fungi, loss of cytoplasm in injured hyphae is restricted by the fast and efficient sealing of the central pores of hyphal cross walls, or septa, by a peroxisome-derived organelle called the Woronin body (Jedd and Chua, 2000; Markham and Collinge, 1987). The Woronin body is only occasionally associated with plugged septa in incompatible hyphal compartments (Fleissner and Glass, 2007). However, a protein required for hyphal fusion in *N. crassa*, SOFT, localizes to septal plugs in incompatible hyphal compartments (Fleissner and Glass, 2007; Fleissner et al., 2005). These data suggest that some components of the hyphal fusion machinery may also be involved in VI. Some VI interactions may also lead to the progressive loss of one of the nuclear types of the heterokaryon (Pittenger and Brawner, 1961); such unstable heterokaryons do not show growth inhibition, hyphal compartmentation, or death. It is likely that the instability of a nuclear partner in a heterokaryon occurs by biochemical and genetic mechanisms distinct from those of VI.

One of the phenotypic aspects associated with VI is vacuolization of incompatible hyphal compartments, which eventually burst (Glass and Kaneko, 2003). In filamentous fungi, vacuoles contain numerous proteases and degradative enzymes, which are released into the cytoplasm upon lysis of the vacuoles. In *P. anserina*, cell death is associated with the induction of genes involved in autophagy (Baehrecke, 2005; Pinan-Lucarre et al., 2003, 2005, 2007). In other eukaryotic systems, autophagic PCD has been shown to have biochemical and molecular features distinct from those of apoptotic PCD (Bursch, 2001). In *P. anserina*, a strain containing a deletion of *PaATG1*, the *Podospora* orthologue of the *S. cerevisiae* ATG1 gene, which is involved in the early steps of autophagy in yeast, abolished autophagy in *P. anserina*, but VI was not affected (Pinan-Lucarre et al., 2005, 2007). In fact, cell death by VI occurred more quickly in the *ΔPaATG1* mutants, suggesting that the induction of autophagy during VI may have a protective role.

PCD in Filamentous Fungi

Ultrastructural changes in dying cells during VI are consistent with features associated with PCD in multicellular eukaryotes (Konopleva et al., 1999). A number of biochemical assays have been used to assess cellular changes associated with apoptosis in filamentous fungi, including terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) for DNA degradation, annexin-fluorescein isothiocyanate binding for the presence of phosphatidylserine on the outer leaflet of the plasma membrane, and 2,7′-dichlorodihydrofluorescein diacetate for reactive oxygen species (ROS) production. Based on these assays, an apoptosis-like phenomenon occurs in filamentous fungi and in unicellular fungi, such as *S. cerevisiae*, in response to treatment with hydrogen peroxide, phytosphingosine, antifungal proteins (PAF), amphotericin B, and farnesol (Castro et al., 2008; Chen and Dickman, 2005; Cheng et al., 2003; Jin and Reed, 2002; Leiter et al., 2005; Ludovico et al., 2005; Mousavi and Robson, 2004; Semighini et al., 2006) and entry into stationary phase and asexual sporulation in *Aspergillus* sp. (Mousavi and Robson, 2003; Thrane et al., 2004). In *A. nidulans*, resistance to PAF and farnesol was associated with a dominant-interfering mutation in the α-subunit of G protein, $fadA^{G203R}$ (Leiter et al., 2005) or mutations in the Gβ subunit (Δ*sfaD*), respectively (Semighini et al., 2006). Interestingly, in *P. anserina* mutations in the *mod-D* gene, which encodes the α-subunit of a heterotrimeric G protein orthologous to *fadA*, suppress growth defects associated with some VI interactions. These observations suggest a connection between PCD induced by exposure to environmental toxins and VI.

In *N. crassa*, the cytology of hyphal fusion compartments between incompatible strains that differ in *het* specificity (*mat*, *het-c*, or *het-6*) showed extensive nuclear degradation and TUNEL-positive nuclei (Marek et al., 2003) and elaboration of ROS (Hutchison et al., 2009). In *S. cerevisiae*, ROS and TUNEL-positive nuclei have been associated with PCD in response to a variety of environmental treatments, some of which require a metacaspase gene, *YCA1* (Khan et al., 2005; Madeo et al., 2002; Mazzoni and Falcone, 2008). Caspases are proteases in animal cells that are activated during apoptosis and are involved in proteolytic cleavage of proteins important to the death process. These observations suggest that some aspects of PCD in *S. cerevisiae* are similar to that of metazoan apoptosis (Mazzoni and Falcone, 2008). *N. crassa* and other filamentous ascomycete fungi have two metacaspase orthologues; deletion of either or both of these predicted metacaspase genes had no effect on the frequency of cell death or on the phenotype of VI (Hutchison et al., 2009). These data suggest that even though some cytological events (such as ROS generation and nuclear degradation) are similar between VI and apoptosis, other currently unknown mechanisms are associated with the execution of death during VI.

In addition to nonself recognition between fungal individuals of the same species, nonself recognition also occurs between hyphae of different fungal species, such as between *P. anserina* and *Coprinopsis cinerea*. These interactions were associated with the generation of hydrogen peroxide and death of hyphae (Silar, 2005). It is likely that these mycelial interactions occur without hyphal fusion; fusion is rarely observed between species that are distantly related (Köhler, 1930).

EVOLUTIONARY ASPECTS

Kin selection theory (Hamilton, 1987) provides the most widely accepted explanation for the evolution of allorecognition (Buss and Green, 1985; Buss, 1987; Grafen, 1990; Rousset and Roze, 2007). Fusion between individuals has

potential costs and benefits. Potential benefits include increase in size, with associated increases in reproductive output and survivorship, while the costs involve the potential for competition among reproductive cells (Buss and Green, 1985). If fusion occurs between related individuals, the benefits of fusion may still apply, but the potential costs of reproductive competition are reduced by a fraction proportional to the relatedness with the fusing partner (Buss and Green, 1985). Kin selection theory thus predicts that the cost of fusion decreases as relatedness increases, hence favoring allorecognition.

Although not always explicitly, most explanations for the evolution of VI, and of nonself recognition (allorecognition) in general, have focused on this aspect: VI ensures that somatic growth exclusively benefits closely (clonally) related reproductive cells and thereby restricts the spread of selfish nuclei (or selfish cell lineages in organisms other than fungi) and also of harmful cytoplasmic elements, such as mycoviruses (for examples, see Hartl et al., 1975; Buss, 1987; Nauta and Hoekstra, 1994; Paoletti et al., 2007). Although this explanation makes intuitive sense, theoretical models have pointed out some complications. One complication of the evolution of allorecognition, first noted by Crozier (1986), is how selection directly affects the recognition genes used to estimate overall relatedness. If the short-term costs of VI are larger than the short-term benefits (or, relatively equivalent, if somatic fusion is reciprocally beneficial), positive-frequency-dependent selection on common *het* alleles will eliminate the genetic polymorphism required for VI. Furthermore, even though allorecognition can evolve as a protection against selfish nuclei or other cytoplasmic elements (Hartl et al., 1975), the number of vcgs in filamentous fungi is difficult to explain (Nauta and Hoekstra, 1994, 1996).

Patterns of *het* Gene Polymorphism and Signs of Balancing Selection

During the last decade, several studies have characterized the evolution of *het* loci. Some general features are emerging from these investigations. One remarkable attribute of *het* loci is that they are often completely unrelated between species and, furthermore, that homologues that function as *het* loci in one species can lack such function in other species (for examples, see Kerenyi et al., 2006; and Saupe et al., 2000). Saupe et al. (2000) found that the homologue of the *Neurospora het-c* gene probably does not function as a *het* locus in *P. anserina*, most likely because this species lacks the required polymorphism associated with nonself recognition. A second feature is that many *het* loci have other functions in addition to their role in VI. For example, in several ascomycete fungi, the two mating-type idiomorphs also confer VI (reviewed by Shiu and Glass, 1999; see above). Another example is the *het-c* locus of *P. anserina*, which also has a function during sexual reproduction (Saupe et al., 1994). A third feature of VI is that balancing selection maintains diversity of *het* loci and positive selection generates diversity in these genes in ascomycete species (Glass and Kaneko, 2003; Saupe, 2000; Paoletti et al., 2007). In *Neurospora* and related genera, balancing selection has even resulted in transspecies polymorphism (Wu et al., 1998; Powell et al., 2001, 2007); the three alleles of *het-c* were also found in nearly equal frequencies in population samples. Finally, some regions of the *het-c* sequence showed a high frequency of nonsynonymous substitutions. These features all strongly suggest that balancing selection has operated to maintain allelic diversity at *het-c*. Similarly, alternate *un-24/het-6* haplotypes were found in equal frequency in an *N. crassa* population isolated from a Louisiana sugarcane field along with a high frequency in nonsynonymous substitutions in the *het-6* alleles (Smith et al., 2000a, 2000b). However, balancing selection is not a general feature of all *het* loci (see Milgroom and Cortesi, 1999).

Structural features of *het* loci may guarantee a high mutation supply. For example, the *het-d* and *het-e* loci, involved in nonallelic VI in *P. anserina*, consist of repeated units (Paoletti et al., 2007). The high copy number of these repeats increases the probability of a mutation in one of the copies. In combination with positive selection and concerted evolution, this may enhance the mutation rate of this repeated element. RIP (repeat-induced point mutations), a fungus-specific hypermutation process acting specifically on repeated sequences, might further enhance the mutation supply of repeated sequences (Paoletti et al., 2007).

In summary, *het* genes are highly polymorphic, often have different cellular functions in addition to their role in VI, display signs of positive and balancing selection, and can have structural features that guarantee a high mutation supply.

The Consequences of Fusion and VI

As indicated earlier, somatic fusion has fitness consequences for both partners in an encounter. These fitness consequences can be caused by genetic differences between the partners or by nongenetic effects of fusion. A possible advantage caused by genetic differences between partners may derive from genetic complementation, leading to chimeric vigor (Buss, 1999). Furthermore, especially relevant for purely asexual fungi, somatic fusion may facilitate recombination via parasexuality (Pontecorvo, 1958). However, VI severely restricts the potential for heterokaryon formation, and therefore, the significance of parasexual recombination probably does not lie in recombination between divergent asexual clones as was suggested initially (Haldane, 1955). However, a recent paper has shown that parasexuality may nevertheless accelerate adaptive evolution, because mutation may generate sufficient variation to make genetic recombination effective (Schoustra et al., 2007).

Depending on how the reproduction between the two partners is divided, somatic fusion can also be advantageous for only one partner, at a cost to the other. Effectively, this means that one of the two partners parasitizes the other (Buss, 1987; Grosberg, 1988). Parasitic nuclear genes have been found in *N. crassa* (Pittenger and Brawner, 1961). The parasitic allele has a transmission advantage in the presence of its nonparasitic homologue but lowers the fitness of the individual in homozygous condition. Parasitism may be conditional on realized investment; if a mycelium has reached a certain size or invested in the production of sexual structures, fusion bears the risk that the investment will be exploited by another genotype ("resource plundering" [Debets and Griffiths, 1998]).

The second category is nongenetic consequences of fusion, which can be measured as the fitness outcomes of self fusion, i.e., fusion between genetically identical individuals. If the average costs and benefits of fusion cancel out, self fusion is neutral. A second possibility is that fusion is reciprocally beneficial, resulting in synergy. Several possibilities exist for synergy. Fusion between different individuals results in an instantaneous increase in the number of somatic cells. This can be mutually advantageous if the initiation of reproduction depends upon a critical size of an individual, which can be reached faster via fusion. If the risk of mortal-

ity is size dependent, an increase in size via fusion reduces this risk for both partners. Another potential advantage of fusion is that spatially separated substrates are more efficiently utilized (Buss, 1982; Rayner et al., 1984). Furthermore, if VI is costly, for example during barrage formation, fusion is automatically reciprocally beneficial, *relative* to VI.

The last possibility is that fusion results in a net loss of fitness. For example, fusion between an individual infected with a harmful horizontally transmitted virus and an uninfected individual will cause a net loss of fitness. Mycoviruses and other harmful cytoplasmic elements are widespread among fungi, so potentially they are a potent selective force. VI has indeed been shown to reduce the transmission of parasitic cytoplasmic elements (Brasier, 1986a, 1986b; Buck and Brasier, 2002; van Diepeningen et al., 1997), although it does not always fully prevent it (Caten, 1972; Anagnostakis and Day, 1979; Anagnostakis, 1983; Debets et al., 1994). For the invasive pathogen *Ophiostoma novo-ulmi*, strong evidence has been obtained that the specific acquisition of VI genes (and a MAT allele, which enabled sexual reproduction) from the related species *O. ulmi* has restricted the spread of cytoplasmic viruses (Paoletti et al., 2006).

Assumptions about the Consequences of Self Fusion Determine the Outcome of Theoretical Studies

The assumption about the consequences of somatic fusion with self is the main determinant for the evolutionary stability of kin recognition polymorphisms in theoretical models (Aanen et al., 2008). If self fusion is completely neutral, it is hard to explain the maintenance of kin-conditional fusion. It has been suggested that VI is selectively neutral (Nauta and Hoekstra, 1996; Saupe, 2000). However, this seems unlikely, given the widespread occurrence of allorecognition in other organisms, and also at other levels of biological organization.

The potential for selfish behavior of nuclei or cytoplasmic elements may possibly explain the evolution of conditional fusion (Hartl et al., 1975; Nauta and Hoekstra, 1994, 1996; Muirhead et al., 2002). However, theoretical modeling assuming a homogeneous population structure fails to explain the extreme level of polymorphism that is observed in most fungal species (Nauta and Hoekstra, 1994, 1996). Incorporating population subdivision, which means that competition occurs locally and not globally, may result in a higher total number of recognition alleles, but this remains to be studied.

If between-individual fusion is mutually advantageous (or, relatively equivalently, if VI is costly), VI is more difficult to explain. Crozier (1986) addressed this problem for sessile marine invertebrates, which behave aggressively to nonself, but the problem also applies to other allorecognition systems (see Rousset and Roze, 2007). Individuals bearing common *het* genes fuse more frequently and hence have a higher fitness than individuals bearing rare *het* genes. This means that *het* alleles are under positive-frequency-dependent selection and that *het* diversity will decrease. However, cheating genes are then likely to evolve in the population and become associated with common *het* alleles (Grafen, 1990). Under these circumstances, rare *het* alleles are more informative of close relatedness, which can lead to a lower frequency of cheater genes among bearers of rare alleles. This gives rare alleles an indirect advantage, which could stabilize the kin recognition system. Rousset and Roze (2007) have recently modeled the balance between these two effects, the direct benefit of a common marker and the indirect benefit of a rare marker. They concluded that under many circumstances the direct benefit of a common marker prevails, and, therefore, allorecognition polymorphisms disappear.

A possible solution for this theoretical problem, first suggested by Crozier (1986), is that selection for functions other than allorecognition maintains polymorphism (Crozier, 1986; Rousset and Roze, 2007). Some of the other functions of *het* loci in addition to allorecognition may lead to negative-frequency-dependent selection and thereby maintain diversity. One of the best examples is provided by the two mating-type alleles that also function as *het* alleles in several ascomycete fungi (see above). The mating-type genes are under strong balancing selection, since in heterothallic species both mating types are required for successful sexual reproduction. Genes associated with pathogen defense may also have been recruited for allorecognition functions (Fedorova et al., 2005), such as proteins involved in fungal allorecognition that share the HET domain. Parasite coevolution could stabilize polymorphism through negative-frequency-dependent selection, analogous to the major histocompatibility complex region in vertebrates. As mentioned above, the recently discovered homologue of the *Neurospora het-c* gene in the bacterium *P. syringae* also suggests that *het* genes may be involved in interspecies interactions (Wichmann et al., 2008).

It should be noted that if external selective forces are responsible for the *stability* of *het* polymorphism, it must still be beneficial to condition the *response* (cell death) on allotype (Grosberg and Quinn, 1989). If this were not the case, selection would work against VI. The fact that a recognition domain and an effector domain can be distinguished in the predicted products of most *het* loci (Paoletti and Clave, 2007; this chapter) supports this idea. In some cases, the effector is not even linked to the recognition locus (e.g., the *tol* locus, which is required for mating-type incompatibility in *N. crassa*).

Finally, if somatic fusion is associated with costs, selection will favor rare *het* alleles, because carriers of a rare allele will fuse less often. This will result in the maintenance of recognition polymorphism (Muirhead et al., 2002). It has been questioned, however, why the ability to fuse would not be lost altogether under these circumstances (Rousset and Roze, 2007). A possible reason is that fusion *within* the individual still provides an advantage (Nauta and Hoekstra, 1994). The significance of intraindividual fusion has indeed been shown by using a mutant of *N. crassa* that lacks the capacity to anastomose and exhibits slower and aberrant growth (Xiang et al., 2002) (see chapter 19). Similar observations have been made for other fungi (A. J. M. Debets, unpublished observations). Intraindividual fusion is believed to serve intraorganismal communication, redistribution of water and nutrients, and general homeostasis within a mycelium (Rayner et al., 1984).

Secondary Consequences of VI

So far, the consequences of VI that have been considered are believed to be primarily responsible for any selective explanations for the evolution of VI, such as a restriction of the spread of selfish nuclei and cytoplasmic elements. However, in addition to these primary effects of VI, there are other secondary consequences of VI. A consequence of nonallelic VI in the pseudohomothallic species *N. tetrasperma* and *P. anserina* occurs in the case of outcrossing, where a fraction of the spores are inviable due to the presence of incompatible nuclei in ascospore progeny. This process promotes inbreeding and may contribute to speciation.

In the pseudohomothallic species *P. anserina*, some loci involved in nonallelic somatic incompatibility also have a

function in sexual reproduction (Bernet, 1992). The same gene interactions that lead to VI between different mycelia lead to PCD during the transformation of vegetative tissue into reproductive tissue (Bernet, 1992). These loci are under selection to coevolve within an individual, and since *P. anserina* is secondarily homothallic, particular loci become associated for extended periods of time. This extended intraindividual coevolution between allelic pairs could also be a mechanism to stabilize polymorphisms (see above).

van der Gaag et al. (2003) found a possible link between VI and meiotic drive in *P. anserina*. They suggested that this link may be accidental, because of the expression of VI at the wrong moment (during the sexual cycle). However, if balancing selection acts on *het* loci, spore killer genes linked to different alleles might "hitchhike" along with these alleles (van der Gaag et al., 2003).

CLOSING REMARKS

VI in fungi as a paradigm for allorecognition in genetically tractable simple eukaryotic species holds great promise to gain a better understanding of the general principles that govern the evolution of nonself recognition systems. It also represents an attractive model for dissecting PCD mechanisms in fungi. Recent efforts have led to the emergence of a few important principles: first, the essentially nonallelic nature of the *het*-gene interactions, and second, the near systematic involvement of HET domain proteins. But although the main players are now characterized in a number of systems—and sometimes to a great extent—the exact modalities of the recognition step and its translation into a cell death reaction remain unclear. It is now also clear that *het*-genes are, as a rule, under a particular evolutionary regimen and experience balancing selection and positive diversifying selection. However, the exact nature of the selective forces that lead to these evolutionary signatures is still largely unknown. In that sense, the hypothesis that *het* systems are in some way related to heterospecific recognition and/or host defense mechanisms is particularly appealing.

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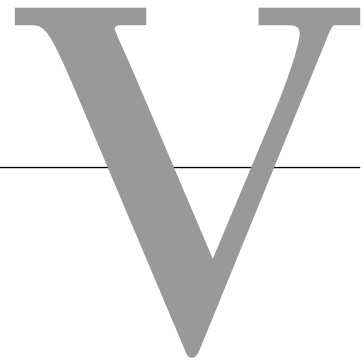
METABOLISM



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Glucose

MARGARET E. KATZ AND JOAN M. KELLY

Glucose can provide a primary source of energy and metabolic intermediates for eukaryotic microorganisms. The yeast *Saccharomyces cerevisiae* has been considered the paradigm for eukaryotic cells. In yeast, extracellular glucose is transported by a range of high- and low-affinity transporters, and transport is regulated in response to carbon status. Nutrient sensing and signaling via pathways involving hexokinases, glucose transporter-like proteins that lack transport activity, and G-protein-coupled receptors have been characterized in yeast. Glucose is the preferred energy source; the genes that are required for the use of alternative carbon sources are not transcribed when glucose is available, and the key components of this transcriptional repression mechanism, including the Mig1p repressor protein, the Ssn6p-Tup1p corepressor complex, and the Snf1p kinase, have been extensively studied. Studies of multicellular filamentous fungi are only at an embryonic stage, but it is becoming clear that the situation is considerably more complex in filamentous fungi than in yeast, probably due to the restricted metabolic capacity of yeast due to strong selection for anaerobic fermentation of sugars to ethanol, rather than aerobic metabolism via the Krebs cycle as in filamentous fungi. A large number of sugar transporters have been predicted from the filamentous fungal genome projects, but functional data exist for only a small fraction of these proteins in Ascomycetes (*Aspergillus niger*, *Aspergillus nidulans*, *Neurospora crassa*, *Trichoderma harzianum*, and *Tuber borchii)* and Basidiomycetes (*Amanita muscaria* and *Uromyces fabae*). Similarly, the studies that have been performed with a few filamentous fungi have revealed that glucose sensing and signaling, as well as carbon catabolite repression, are achieved by different molecular mechanisms, not only between yeast and the filamentous fungi, but also between groups of filamentous fungi. Here we aim to review what is known about the regulation of glucose uptake and metabolism in filamentous fungi and to highlight the similarities with and differences from mechanisms in yeast.

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GLUCOSE TRANSPORT

Fungal Glucose Transporters

Early work on glucose transport in filamentous fungi showed that there are two transport systems (Mark and Romano, 1971; Scarborough, 1970; Schneider and Wiley, 1971). Fungal glucose transporters are classified as high-affinity transporters if the K_m for glucose is in the micromolar range (e.g., <50 μM for high-affinity transporters from filamentous fungi) and low-affinity transporters if K_m for glucose is in the millimolar range. High-affinity glucose transporters are usually expressed during carbon starvation and are repressed by glucose. Low-affinity transporters are typically expressed when the concentration of glucose, or other hexoses, is high.

The identification and characterization of genes encoding hexose transporters in *S. cerevisiae* have shown that the assumption that there is one high-affinity transporter and one low-affinity transporter is incorrect. Multiple transporters contribute to each transport system (Özcan and Johnston, 1999). All of the fungal glucose transporters that have been characterized to date belong to the "Sugar (and other) Transporters" clan of the major facilitator superfamily (http://pfam.sanger.ac.uk/family?acc=PF00083). Major facilitator superfamily sugar transporters contain 12 transmembrane domains, which are thought to have arisen through a duplication of a six-transmembrane unit in prokaryotes. There is a longer cytoplasmic loop between the two sets of six transmembrane domains (Marger and Saier, 1993).

Glucose Transport in *S. cerevisiae*

S. cerevisiae contains a large number of proteins that can transport glucose across the yeast cell membrane, 17 of which (Hxt1 through Hxt11p, Hxt13 through Hxt17p, and Gal2p) belong to the yeast glucose transporter family (Fig. 1). These transporters differ in specificity for glucose, fructose, mannose, and galactose. They also differ in regulation in response

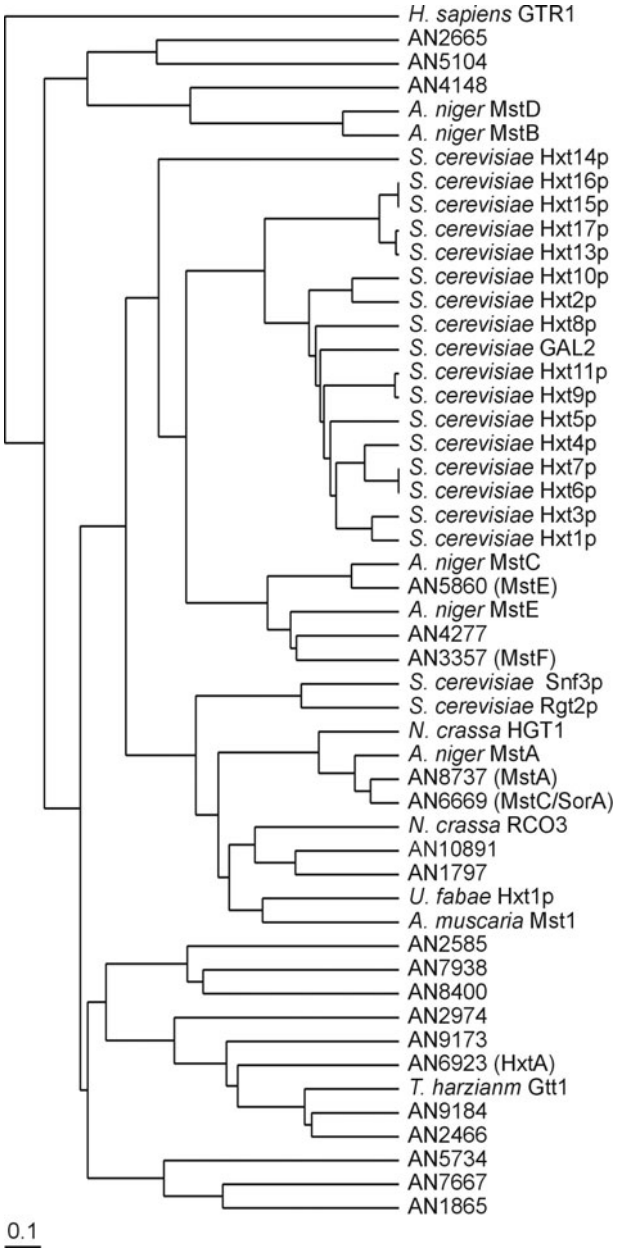

FIGURE 1 Phylogenetic tree of putative sugar transporters in *A. nidulans*. *A. nidulans* (AN) putative proteins that show similarity to fungal hexose transporters have been characterized in *A. muscaria* (Mst1), *A. nidulans* (HxtA and MstE), *A. niger* (MstA), *N. crassa* (HGT1 and RCO3), *S. cerevisiae* (Hxt1p through Hxt11p, Hxt13p through Hxt17p, and Gal2p). *T. harzianum* (Gtt1), and *U. fabae* (Hxt1p). The rooted tree was constructed using CLUSTAL (Thompson et al., 1994), PROTDIST and KITSCH (Felsenstein, 1996) through Biomanager at the Australian National Genome Information Service ((http://www.angis.org.au), and TREEVIEW (Page, 1996). The amino acid sequences used to construct the tree were obtained from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/), NCBI (http://www.ncbi.nlm. nih.gov/), and the *Aspergillus* Comparative Database (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html). The human glucose transporter GTR1 was included as an outgroup.

to glucose concentration, carbon starvation, osmotic pressure, and physiological status (Özcan and Johnston, 1999). In addition, yeast possesses two members of the yeast glucose transporter family, Snf3p and Rgt2p, which act as glucose sensors but do not transport glucose. The role of these two proteins in glucose sensing will be discussed in "Glucose Transporter Homologues" below.

Deletion of transporter genes *HXT1–7* prevents growth on glucose (Özcan and Johnston, 1999). However, Wieczorke and colleagues (1999) found that a strain that lacked *HXT1–17* and *GAL2* was still able to take up glucose. Deletion of an additional three maltose permease genes (*AGT2*, *MPH2*, and *MPH3*) was required to completely abolish glucose transport. Even this strain, lacking Hxt1–17p, Gal2p, Agt2p, and Mph2–3p, was able to grow in medium containing glucose as a sole carbon source if the *SNF3* regulator was absent, indicating that Snf3p represses one or more additional genes whose products act as hexose transporters (Wieczorke et al., 1999).

Glucose Transporters in Filamentous Fungi

Identification of Glucose Transporters

The genomes of filamentous fungi contain a large number of genes that are similar to yeast hexose transporter genes. *A. nidulans* contains 109 genes and *N. crassa* contains 45 genes which are predicted to encode proteins belonging to the sugar transporter clan of the major facilitator superfamily. In some cases these proteins are known to be involved in the transport of molecules other than sugars (e.g., AN1138 and quinate permease).

Only a few genes encoding glucose transporters have been analyzed in filamentous fungi (Table 1). It is likely that these genes represent only a small sample of the hexose transporter genes present in the genomes of multicellular fungi. The *A. nidulans* genome contains many proteins similar to known fungal glucose transporters. Phylogenetic analysis of some of these *A. nidulans* proteins is shown in Fig. 1. The tree shows that most of the yeast glucose transporters form a single group that is separate from the transporters identified in filamentous fungi. This suggests that yeast transporters may be adapted to a very specific set of environmental conditions, nutrient sources, and/or the unicellular state and may not be representative of the diversity of glucose transporters found in other fungi.

The *N. crassa rco-3* gene encodes a putative glucose transporter, but characterization of *rco-3* mutants suggests that, like *S. cerevisiae* Snf3p and Rgt3p, RCO-3 is involved in glucose sensing rather than glucose uptake (Madi et al., 1997). The role of RCO-3 in glucose sensing is covered in "Glucose Transporter Homologues" below.

Functional Analysis of Glucose Transporters

Because fungi possess multiple glucose transporters, it can be difficult to study the properties of the proteins encoded by individual genes. In some cases, such as the *N. crassa hgt-1*, *A. niger mst*A, and *A. nidulans mstE* genes, it has been demonstrated that disruption of a single transporter gene alters glucose uptake (Xie et al., 2004; vanKuyk et al., 2004; Forment et al., 2006). One strategy that can be used to demonstrate that a gene encodes a glucose transporter is to express the gene in a strain of *S. cerevisiae* that is unable to transport glucose. When the *U. fabae HXT1* gene is inserted in a yeast expression vector, it can complement the *S. cerevisiae hxt1–7*Δ deletion mutations and restore the ability to use glucose as a carbon source (Voegele et al., 2001). Using a similar strategy,

TABLE 1 Characterization of glucose transporters in filamentous fungi

Species	Protein	Location	Complementation in yeast	Affinity for glucose	Expression pattern	Phenotype of gene disruption mutant	Reference
A. muscaria	AmMST1	Hyphae	Yes	Unknown	Constitutively expressed in hyphae, upregulated in mycorrhizas and glucose concn of >5 mM	Unknown	Nehls et al., 1998
A. nidulans	HktA	Young cleistothecia and hyphae	No	High (proposed)	Expressed in response to carbon starvation and sexual development	No effect on growth or sexual development	Wei et al., 2004
A. nidulans	MstE	Conidial and hyphal membrane, septae, and vacuole	Not tested	Low	High during growth in glucose (and other repressing carbon sources)	No difference in growth; loss of low-affinity glucose transport	Forment et al., 2006
A. nidulans	SorA	Unknown	Not tested	High	Not tested	Loss of high-affinity glucose uptake, sorbose resistant	MacCabe et al., 2003; MacCabe et al., unpublished
A. niger	MstA	Unknown	Partial	High	High during carbon starvation and in presence of poor carbon sources. Repressed by high glucose concn.	No difference in growth; increased expression of *mstC*, reduced expression of *mstF*; reduced affinity for glucose	vanKuyk et al., 2004 Jørgensen et al., 2007
N. crassa	RCO-3	Unknown	Not tested	None?	Unknown	Reduced high- and low-affinity glucose uptake, defects in glucose repression, conidiation in the presence of glucose; sorbose and 2DOG resistant	Madi et al., 1997
N. crassa	HGT-1	Unknown	Not tested	High	High during carbon starvation, repressed by glucose	Reduced rate of high-affinity glucose uptake	Xie et al., 2004
T. harzianum	Gtt1	Unknown	No	High	High during carbon starvation, repressed by glucose.	Unknown	Delgado-Jarana et al., 2003
T. borchii	TBHXT1	Hyphae	Yes	High	Highest at 3 mM glucose concn and after prolonged starvation, lower at low and high glucose concn	Unknown	Polidori et al., 2007
U. fabae	Hxt1p	Haustorial plasma membrane	Yes	Medium	Unknown	Unknown	Voegele et al., 2001

vanKuyk and colleagues showed that the *A. niger mstA* gene can partially complement the growth defect of an *S. cerevisiae hxt1–7Δ gal2Δ* deletion mutant (vanKuyk et al., 2004). Nehls and colleagues showed that expression of *Amanita muscaria* AmMST1 could complement the growth defect of a *Schizosaccharomyces pombe* mutant unable to import glucose (Nehls et al., 1998). An alternative strategy was used by Delgado-Jarana and colleagues, when it was found that the *T. harzianum gtt1* gene did not complement *S. cerevisiae* mutants unable to use glucose (Delgado-Jarana et al., 2003). They showed that overexpression of the *gtt1* gene increased glucose uptake in *T. harzianum*. The *A nidulans hxtA* gene, which shows a high degree of similarity with *gtt1* (Fig. 1), also failed to restore glucose utilization to an *S. cerevisiae* hexose transport mutant (Wei et al., 2004).

Substrate Specificity of Transporters

Like yeast sugar transporters, the transporters from filamentous fungi have been shown to have multiple substrates. *A. niger* MSTA has a very high affinity for glucose (K_m, 25 μM) and mannose (K_m, 60 μM) but transports xylose and fructose with lower affinity (K_m, 300 μM and 4 mM, respectively) (vanKuyk et al., 2004). Experiments carried out with *S. cerevisiae* showed that *U. fabae* Hxt1p has a higher affinity for glucose (K_m, 360 μM) than for fructose (K_m, 1 mM). Mannose is also a substrate for Hxt1p, but galactose, trehalose, maltose, and sucrose are not (Voegele et al., 2001). The *T. borchii* TBHXT1 transporter has also been shown to have a higher affinity for glucose (K_m, 38 μM) than fructose (K_m, 16 mM) (Polidori et al., 2007). *A. niger* MstA, *U. fabae* Hxt1p, and *T. borchii* TBHXT1 have all been shown to be sugar/H^+ symporters.

Tissue-Specific Expression of Glucose Transporters

Several of the hexose transporters that have been characterized show tissue-specific expression. Voegele et al. (2001) identified a hexose transporter (Hxt1p) from the biotrophic plant pathogen *U. fabae*. They showed that the *hxt1* gene is expressed only in haustoria and the protein is found on the haustorial plasma membrane, which suggests that the Hxt1p transporter has a specific role in the uptake of sugars from plant cells. Expression of the AmMST1 glucose transporter is upregulated in the ectomycorrhizas that form when *A. muscaria* grows in association with plant roots, which suggests that it is also involved in the acquisition of plant-derived nutrients (Nehls et al., 1998). In contrast, the *T. borchii* gene, *Tbhxt1*, which encodes a high-affinity transporter, was not expressed at higher levels in ectomycorrhizas (Polidori et al., 2007). Another example of a transporter that shows tissue-specific regulation is the *A. nidulans* HxtA transporter, which is expressed during sexual development (Wei et al., 2004).

Regulation of Glucose Transport

Carbon Regulation

Most of the genes encoding fungal glucose transporters are either upregulated or repressed by glucose. The DNA-binding protein that mediates glucose repression in *Aspergillus* is encoded by the *creA* gene (Bailey and Arst, 1975; Dowzer and Kelly, 1991; Kulmburg et al., 1993) and is discussed further in "Mutations Leading to Carbon Catabolite Derepression" below. MacCabe et al. (2003) showed that the high-affinity glucose uptake system, which is repressed by glucose in wild-type strains, is derepressed in *A. nidulans* strains lacking a functional copy of the *creA* gene. In contrast, the low-affinity system is not induced by glucose in *creA* null mutants. Analysis of individual transporter genes has provided support for these findings. The *A. niger mstA* gene, which encodes a high-affinity glucose transporter, is expressed during carbon starvation and is repressed by high concentrations of glucose. In a *creA* null mutant, *mstA* expression is higher when glucose and other sugars are present but expression in response to carbon starvation is greatly reduced (vanKuyk et al., 2004). The *A. nidulans mstE* gene, which encodes a low-affinity glucose transporter, shows a completely different pattern of expression. The *mstE* transcript is at high levels during growth on glucose, fructose, mannose, sucrose, and other carbon sources that trigger CreA-mediated carbon catabolite repression and is low during growth on nonrepressing carbon sources (Forment et al., 2006). In a *creA30* loss-of-function mutant, expression of *mstE* was abolished.

Many of the high-affinity glucose transporters have been shown to be expressed at very high levels during carbon starvation. The *A. nidulans xprG* gene encodes a putative transcriptional activator that regulates the response of extracellular proteases to carbon starvation (Katz et al., 2006). Microarray analyses and glucose uptake assays suggest that XprG is also involved in the regulation of high-affinity glucose transport in *A. nidulans* (K. Sue and M. E. Katz, unpublished observations).

pH Regulation

The regulation of transporter gene expression by pH has also been investigated. Expression of the *A. niger mstA* gene is highest at pH 6 and lower at both pH 4 and pH 8 (vanKuyk et al., 2004). The transcription factor encoded by the *A. nidulans pacC* gene controls gene expression in response to environmental pH (Tilburn et al., 2005). Truncation of PacC, which has been shown to result in constitutive expression of PacC-regulated genes, led to a significant increase in *mstA* transcript levels (vanKuyk et al., 2004). The *T. harzianum gtt1* gene has also been shown to be regulated in response to pH, but in contrast to the *A. niger mstA* gene, expression was highest at acidic pH (Delgado-Jarana et al., 2003).

METABOLISM OF GLUCOSE IN FILAMENTOUS FUNGI

Glucose is a monosaccharide that is used to fuel cellular respiration in a range of organisms. Most aerobic filamentous fungi metabolize glucose primarily via oxidative phosphorylation, releasing carbon dioxide, water, and energy through a highly conserved series of reactions, although some can also ferment glucose to ethanol (Colvin et al., 1973), whereas *S. cerevisiae* preferentially ferments glucose to ethanol. Glucose may be available in the environment either directly or as the result of the breakdown of disaccharides and polysaccharides including sucrose, starch, and cellulose, or intracellularly, glucose can be generated de novo by gluconeogenesis (chapter 22).

Glycolysis and the Pentose Phosphate Pathway

When glucose is abundant, it is converted to pyruvate with the release of ATP, as shown generically in Fig. 2, although not all glucose is converted to pyruvate, as intermediates may be required in other pathways. A body of early research on each individual enzyme of glycolysis in a number of filamentous fungi exists, with each identified either by enzymatic, genetic, molecular biological, or comparative genomic means (Blumenthal, 1965). Examples

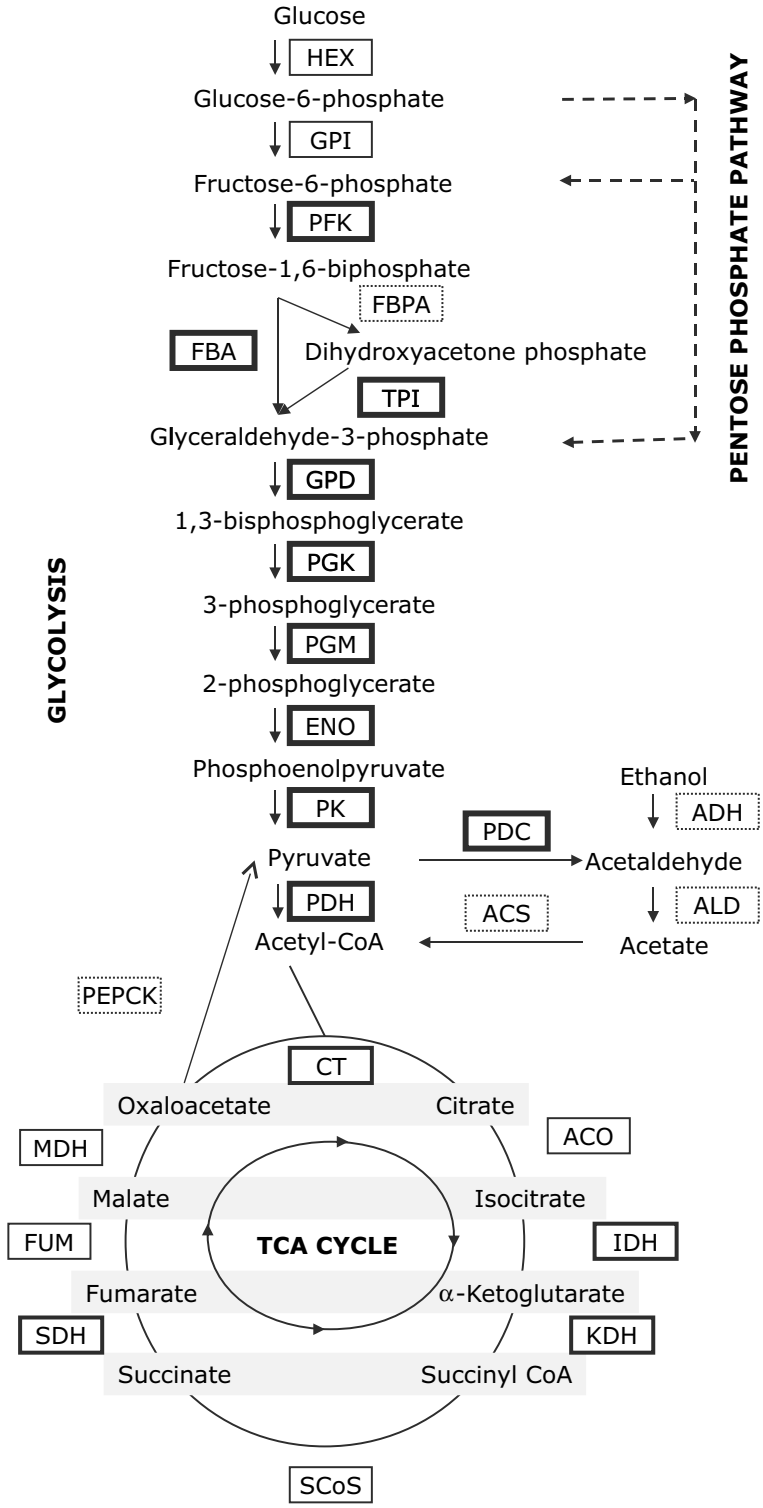

FIGURE 2 Enzymes of the glycolytic pathway and TCA cycle. In *A. oryzae*, genes encoding enzymes indicated in a solid box were increased, and those indicated in a dotted box decreased, in mycelia grown in glucose-rich compared to glucose-poor media (Maeda et al., 2004). Abbreviations: HEX, hexokinase/glucokinase; GPI, phosphoglucose isomerase; PFK, phosphofructokinase; FBA, fructose bisphosphatase; FBPA, fructose bisphosphate aldolase; TPI, triose phosphate isomerase; GPD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, phosphoenolpyruvate kinase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase I; ALD, acetaldehyde dehydrogenase; ACS, acetyl-CoA synthase; PDH, pyruvate dehydrogenase; CT, citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase; KDH, α-ketoglutarate dehydrogenase; SCoS, succinyl-CoA synthase; SDH, succinate dehydrogenase; FUM, fumarate dehydratase; MDH, malate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase.

include hexokinase (Dunn-Coleman and Pateman, 1979; Flipphi et al., 2003; Klingmuller and Truper, 1965; Medina and Nicholas, 1957a, 1957b; Panneman et al., 1998; Ruijter et al., 1996), glucose-phosphate isomerase (Murayama and Ishikawa, 1975), aldolase (Mattoo and Roo, 1974), triosephosphate isomerase (McKnight et al., 1986), glyceraldehyde phosphate dehydrogenase (Punt et al., 1991), phosphoglycerate kinase (Clements and Roberts, 1985; Van Solingen et al., 1988; Vanhannen et al., 1989), phosphoglycerate mutase (Dunn-Coleman and Pateman, 1979; Johnson and Price, 1988; McAleese et al., 1988), enolase (Fischer et al., 1995; Kuwana and Tanaka, 1987; Machida et al., 1996; Toda et al., 2001; Verma et al., 1987), and pyruvate kinase (De Graaff et al., 1988, 1992; De Graaff and Visser, 1988; Kester et al., 1988; Kuwana and Tanaka, 1987; Schindler et al., 1993; Tsao and Madley, 1975).

Glucose can also be metabolized into pyruvate by the pentose phosphate pathway. The pentose phosphate pathway, which both acts in the conversion of hexose sugars and regenerates NADPH, is an alternative to glycolysis and relies on glycolysis to generate essential metabolites. Mutants that affect the pentose phosphate pathway have been isolated in *A. nidulans* (Hankinson, 1974). In *Penicillium chrysogenum*, the flux through the pentose phosphate pathway increases relative to the flux through glycolysis when the metabolism shifts from rapid growth without penicillin production to slow growth and high penicillin production (Jorgensen et al., 1995).

The Krebs Cycle

In aerobic fungi, pyruvate is primarily converted to acetyl coenzyme A (acetyl-CoA) via the pyruvate dehydrogenase complex (Bos et al., 1981; Harding et al., 1970; Song et al., 1978), and then to carbon dioxide and water, with the release of energy, via the Krebs cycle (Chandra and Shanmugasundaram, 1961; Flavell and Woodward, 1970a, 1970b). This is the key difference between the majority of filamentous fungi and *S. cerevisiae*, in that *S. cerevisiae* prefers fermentation at high glucose concentrations and represses genes for the tricarboxylic acid (TCA) cycle.

Genomic and Metabolic Flux Analyses

An integrated approach to the identification of metabolic pathways and networks, their regulation, and metabolic flux through the cycles has proved fruitful. For example, a stoichiometric model of central carbon metabolism was described using available information on *A. niger* metabolism, even before the fully annotated genome was available, by the integration of genomic, biochemical, and physiological data as well as extrapolation from closely related species (David et al., 2003). The primary metabolism of *P. chrysogenum* was also analyzed by advanced nuclear magnetic resonance measurements (Van Winden et al., 2003). David and colleagues (David et al., 2005, 2006) have reconstructed complete metabolic networks for *A. nidulans* growing on glucose, glycerol, and ethanol and shown that the shift from glucose to ethanol results in increased gluconeogenesis and decreased activity in the glycolytic and pentose phosphate pathways. Further, using ^{13}C-metabolic flux analysis, they have assessed the effects of the absence of CreA (see below) on metabolic flux and by calculation of the ratio between the enzymatic activity of key enzymes at the branch point of the glycolysis and pentose phosphate pathways found a 20% reduction through the pentose phosphate pathway under glucose conditions, whereas the addition of xylose resulted in a 40% increase and a reduction in glycolysis and the TCA cycle (David et al., 2005).

Expressed sequence tag and cDNA microarrays were used to investigate the fate of glucose in *Trichoderma reesei* compared to *S. cerevisiae* (Chambergo et al., 2002; DeRisi et al., 1997). In glucose-grown cultures of yeast and *T. reesei*, critical genes encoding enzymes that control the direction of flow of primary metabolites differ in expression. The expression of genes involved in the TCA cycle and mitochondrial respiration are repressed in yeast, leading to pyruvate being channeled to acetaldehyde, but active in *T. reesei*, leading to the oxidation of pyruvate (Chambergo et al., 2002). A similar approach was taken with *Aspergillus oryzae*, whereby transcription profiles were compared between glucose-rich and glucose-depleted cultures, with genes encoding enzymes of the glycolytic and TCA cycles present at higher levels in glucose-rich cultures than in glucose-depleted cultures (Maeda et al., 2004). Unlike in *T. reesei*, the gene encoding an alcohol dehydrogenase that converts acetaldehyde to ethanol was also more highly expressed in glucose-rich cultures than in glucose-depleted cultures, indicating that fermentation to ethanol could occur alongside aerobic respiration (Maeda et al., 2004). In *N. crassa*, glucose-rich cultures were compared to glucose-starved cultures, and although expression of the gene encoding pyruvate decarboxylase was higher in glucose-rich cultures than in glucose-starved cultures, the expression of the alcohol dehydrogenase that converts acetaldehyde to ethanol was similar in glucose-grown and glucose-starved cultures (Xie et al., 2004). Colvin and colleagues demonstrated that some ethanol is produced in glucose-grown cultures, indicating that some fermentation occurs in addition to respiration via the Krebs cycle (Colvin et al., 1973).

The public availability of the complete genome sequences of many fungi (Galagan et al., 2005; Machida et al., 2005; Nierman et al., 2005) and advances in microarray technology have allowed the integration of in silico approaches to metabolic pathway analysis, to more fully describe the regulation of cellular metabolism. Although microarray analyses in filamentous fungi (Breakspear and Momany, 2007) have proved useful in studies of metabolism, they do not allow direct and indirect effects on transcription to be teased out, and they may not reflect actual enzyme activity, as this can be affected by translational and posttranslational events, including feedback inhibition and inactivation, and protein stability.

GLUCOSE SENSING

Glucose has a wide range of regulatory effects in fungi. Glucose-sensing mechanisms are responsible for detecting glucose levels and triggering these regulatory effects. The sensing of glucose and other sugars is also discussed in chapter 30.

Glucose Analogues

The use of glucose analogues has been used to determine whether glucose sensing in fungi requires uptake and/or further metabolism of glucose. The analysis of analogues that are not transported (e.g., L-glucose), are transported but not phosphorylated (e.g., 6-deoxy-D-glucose), or are phosphorylated but not metabolized further (e.g., 2-deoxy-D-glucose) has shown that phosphorylation of glucose is required to trigger glucose repression in *S. cerevisiae*

(reviewed by Rolland et al., 2001). This suggested that glucose sensing might involve one or more of the hexokinases and glucokinase that phosphorylate glucose. Carbon catabolite repression still occurs in *S. cerevisiae* mutants lacking phosphoglucoisomerase, the enzyme that catalyzes the second step in the metabolism of glucose, confirming that further metabolism of glucose was not necessary (Rose et al., 1991).

As in *S. cerevisiae*, 2-deoxyglucose (2DOG) has been shown to mimic glucose in triggering carbon catabolite repression in the filamentous fungi (Fraser et al., 2001; Hynes et al., 2007; Todd et al., 2003). However, 2DOG does not mimic carbon nutrient sufficiency. Fraser et al. (2001) have shown that AreA-mediated expression of the *fmdS* gene is blocked by carbon starvation even in the presence of 2DOG. Likewise, production of extracellular proteases in response to carbon starvation is only partially repressed by 2DOG (M. Katz, unpublished data). 2DOG also does not stimulate upregulation of the glucose-inducible *A. muscaria* glucose transporter, AmMST1 (Nehls et al., 1998).

Nonmetabolizable, toxic glucose analogues (e.g., sorbose and 2DOG) have been used to select mutants which are defective in glucose transport or phosphorylation in a number of filamentous fungi, including *A. nidulans* (Elorza and Arst, 1971), *Coprinus cinereus* (Moore, 1973), *N. crassa* (Klingmüller, 1967), *P. chrysogenum* (Barredo et al., 1988), and others. Analysis of these mutants has led to the identification of genes encoding glucose transporters including *A. nidulans sorA* (A. P. MacCabe, R. Gonzalez, L.Ventura, J. F. Forment, M. J. A. Flipphi, and D. Ramón, unpublished data) and hexokinases including *A. nidulans glkA* (Flipphi et al., 2003). Numerous studies have employed 2DOG to isolate mutants, which show increased production of fungal enzymes or citric acid (e.g., cellulase in *Trichoderma viride* [Farkas et al., 1981]).

Glucose Sensing in *S. cerevisiae*

In *S. cerevisiae*, three interconnected glucose-sensing mechanisms have been described (reviewed by Rolland et al., 2002, and Santangelo, 2006). These signaling pathways are briefly described in this section. Many of the proteins known to play a role in these pathways have been omitted. In addition to these pathways, which require phosphorylation of glucose, the induction of glycolytic genes requires further metabolism of glucose (Muller et al., 1995).

Hexokinase PII

The role of hexokinases in glucose sensing was confirmed in studies using strains carrying mutations in the three genes encoding sugar-phosphorylating enzymes: *HXK1*, *HXK2*, and *GLK2*. A functional copy of any one of these three genes is sufficient for growth on glucose (Maitra and Lobo, 1983). Hxk2p is required for long-term repression of gene expression by glucose (De Winde et al., 1996). The downstream target of the Hxk2p glucose sensor is the repressor Mig1p (see "CreA Repressor Protein" below).

The role of Hxk2p in triggering carbon catabolite repression has been and still is controversial (Santangelo, 2006). While phosphorylation of glucose is required for glucose repression, the regulatory function of Hxk2p does not require catalytic function (Hohmann et al., 1999). A fraction of Hxk2p is located in the nucleus, where it may play a direct role in glucose repression through interaction with Mig1p (Ahuatzi et al., 2007). There is also evidence that the Hxk2p signal may modulate the activity of the Snf1p kinase that regulates Mig1p activity (Sanz et al., 2000). Snf1p is a homologue of the mammalian cyclic AMP (cAMP)-dependent protein kinase AMPK, which plays an important role in sensing energy status (reviewed by Hardie, 2007).

Glucose Transporter Homologues

The response of glucose-repressible and glucose-inducible hexose transporters is mediated by two glucose transporter homologues, Snf3p and Rgt2p. Snf3p and Rgt2p are similar in structure to the Hxtp and Gal2p proteins (see "Glucose Transport in *S. cerevisiae*" above) but contain several hundred additional amino acids at the C terminus. Snf3p induces expression of high-affinity and medium-affinity glucose transporters in low levels of glucose, and Rgt2p induces expression of low-affinity glucose transporters when high levels of glucose are present (Özcan and Johnston, 1999). Both effects are mediated through Rgt1p, which can act as a repressor or activator of *HXT* transcription depending on glucose concentration (Özcan and Johnston, 1999).

cAMP

A third glucose-sensing system involves activation of AMP-dependent protein kinase A (PKA) through two signaling pathways that activate adenylate cyclase and stimulate production of cAMP in response to glucose: a G-protein-coupled receptor, Gpr1p, and Gα subunit, Gpa2p, and a second pathway involving Ras proteins and Cdc25p (Santangelo, 2006). PKA is involved in the control of glycolysis, gluconeogenesis, cell growth, and stress resistance (Rolland et al., 2002). PKA also regulates *HXT* gene expression through Rgt1p (Kim and Johnston, 2006). It has also been proposed that the signal generated by PKA regulates Mig1p-mediated carbon catabolite repression through modulation of Hxk2p activity (Santangelo, 2006).

Role of Hexokinases in Nutrient Sensing in Filamentous Fungi

Role of Hexokinases in Carbon Catabolite Repression

Hexose-phosphorylating enzymes have been shown to be important in glucose repression in *A. nidulans*, but different enzymes have overlapping roles. *A. nidulans* possesses two genes encoding catalytic hexokinases, *frA* (*hxkA*) and *glkA* (Flipphi et al., 2003; Ruijter et al., 1996). A functional copy of only one of the two genes is required for utilization of glucose as a carbon source. Unlike what is observed in *S. cerevisiae*, in which disruption of only one (*HXK2*) of the three hexokinase genes causes derepression, inactivation of both *frA* and *glkA* is necessary to cause derepression of genes subject to CreA-mediated carbon catabolite repression in *A. nidulans* (Flipphi et al., 2003). This suggests that glucose phosphorylation is required to trigger repression in *A. nidulans*, but neither HxkA nor GlkA appears to have a role similar to *S. cerevisiae* Hxk2p (Table 2).

Role of Hexokinases in Carbon Starvation

In *A. nidulans*, CreA-mediated carbon catabolite repression is triggered by glucose and other preferred carbon sources (see "CreA Repressor Protein" below). Derepression of CreA-regulated genes occurs during growth in medium containing poor carbon sources (e.g., glycerol).

TABLE 2 Comparison of glucose sensing in *S. cerevisiae* and filamentous fungi

Type of glucose sensor	Sensor(s) in yeast	Sensor(s) in filamentous fungi	Role of sensor(s) in filamentous fungi
Hexokinase	Hxk2p	HxkA, GlkA (*A. nidulans*)	No evidence for a direct role of a single hexokinase as in yeast but phosphorylation required to trigger CCR (Flipphi et al., 2003)
		HxkC, HxkD (*A. nidulans*)	No role in CCR; involved in the response to carbon starvation (Bernardo et al., 2007)
Transporter	Snf3p, Rgt2p	RCO-3 (*N. crassa*)	May perform similar function to yeast proteins but structure of RCO-3 differs; could have broader role in CCR than yeast proteins which regulate hexose transporters (Madi et al., 1997)
G-protein-coupled receptor	Gpr1p, Gpa2p (Gα3)	GPR-4, GCNA1–3 (Gα1–3) (*N. crassa*)	G-protein-coupled receptor GPR-4 is involved in cAMP signaling in response to glucose. Unlike in yeast, GPR-4 interacts with Gα1 rather than Gα3, and Gα1 through Gα3 are all involved in nutrient sensing.
		GanB (Gα3) (*A. nidulans*)	Involved in carbon sensing/cAMP signaling in germinating conidia (Lafon et al., 2005)
		BCG3 (Gα3) (*B. cinerea*)	Involved in carbon sensing/cAMP signaling in germinating conidia (Doehlemann et al., 2006)
		GasC (Gα3) (*P. marneffei*)	Required for conidial germination but not involved in nutrient sensing (Zuber et al., 2003)
		Gpa1 (Gα3) (*C. neoformans*)	Involved in cAMP signaling in response to glucose and production of melanin (Alspaugh et al., 1997)
		Gpr4 (*C. neoformans*)	Similar to *S. cerevisiae* Gpr1p but is not involved in glucose signaling (Xue et al., 2006)

However, there are a number of genes, including the regulator of conidiation, *brlA* (Skromne et al., 1995), which are repressed by poor carbon sources as well as by glucose and are only expressed at high levels during carbon starvation. This suggests that carbon catabolite derepression and carbon starvation are not equivalent. Glucose repression of some of the carbon-starvation-inducible genes has been shown to be CreA independent and occurs in strains lacking hexokinase and glucokinase catalytic activity (Katz et al., 2008).

In addition to catalytic hexokinases, some filamentous fungi possess a number of atypical hexokinases (Fig. 3). Genetic and biochemical evidence suggests that these hexokinase-like proteins, which lack some of the highly conserved amino acids in the ATP- and sugar-binding domains, are solely regulatory in function (Bernardo et al., 2007). Analysis of *A. nidulans* HxkC and HxkD (formerly XprF) indicates that these atypical hexokinases modulate the activity of the putative p53-like transcriptional activator, XprG, and are involved in the response to carbon starvation (Katz et al., 1996, 2000, 2006). Most or all of the HxkD protein is located in the nucleus, which is consistent with a direct role in gene regulation (Bernardo et al., 2007). In contrast, HxkC is associated with mitochondria. Mitochondrial hexokinases have been implicated in the control of programmed cell death in both mammals and plants (Kim and Johnston, 2006; Pastorino et al., 2002). Thus, HxkC may play a key role in triggering programmed cell death in response to carbon starvation in filamentous fungi.

Not all species of filamentous fungi appear to possess the signaling pathway defined by HxkC, HxkD, and XprG. The genome of only one basidiomycete, *Ustilago maydis*, has a gene encoding a protein with similarity to the family of Ndt80-like DNA-binding proteins to which XprG belongs (Table 3). In contrast, the zygomycetes contain many members of this class of protein and a correspondingly large number of hexokinases. Even within members of the same species (e.g., *Coccidioides immitis*) or the same genus (e.g., *Aspergillus* and *Fusarium*) the number of hexokinases and Ndt80-like proteins is variable.

Glucose Transporters in Glucose Sensing

Mutations in two glucose transporters from filamentous fungi have been shown to have regulatory effects. The *N. crassa rco-3* gene encodes a protein similar to sugar transporters in *S. cerevisiae* and other fungi (Madi et al., 1997). RCO-3 is longer than most yeast hexose transporters and like Snf3p and Rgt2p has an extended C terminus. However, the RCO-3 C terminus, which has a glutamine-rich region followed by an S/T-rich region, is not similar in sequence to the same regions in the yeast transporter homologues.

The *rco-3* gene was first identified through analysis of an *N. crassa* mutant that could conidiate in submerged cultures and in the presence of high levels of glucose, fructose, glycerol, and xylose (Madi et al., 1994). Both high- and low-affinity glucose transport is altered in mutants lacking the *rco-3* gene (Madi et al., 1997). In addition, derepression of *qa* expression occurs. The many regulatory effects seen in strains lacking functional RCO-3 led to the suggestion that RCO-3 functions as a glucose sensor (Madi et al., 1997).

Disruption of the *A. niger* gene encoding the high-affinity glucose transporter, MstA, results in altered expression of two other genes encoding putative hexose

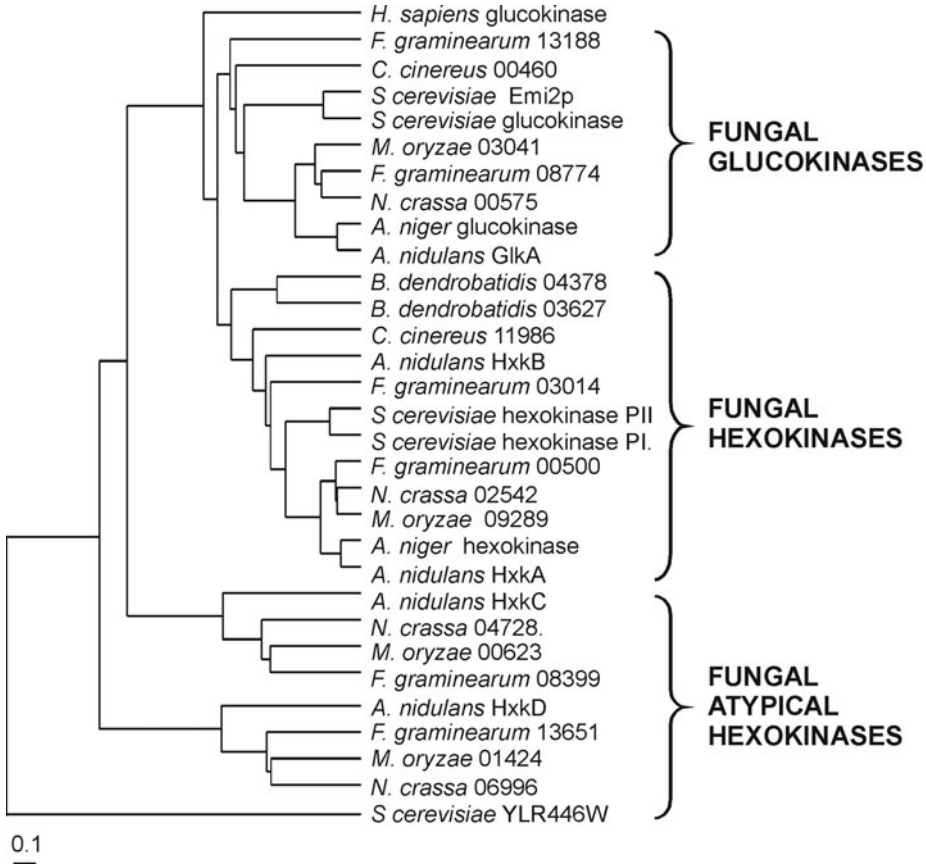

FIGURE 3 Phylogenetic tree of hexokinases and hexokinase-like proteins from *A. nidulans*, *A. niger*, *Batrachochytrium dendrobatidis*, *C. cinereus*, *Fusarium graminearum*, *Magnaporthe oryzae* (formerly *M. grisea*) and *N. crassa*. The rooted tree was constructed with amino acid sequences from the Fungal Genome Initiative at the Broad Institute (http://www.broad.mit.edu/annotation/fgi/) and sequences from *A. niger* (Panneman et al., 1996, 1998) by using CLUSTAL (Thompson et al., 1994), PROTDIST and KITSCH (Felsenstein, 1996) through Biomanager at the Australian National Genome Information Service (http://www.angis.org.au), and TREEVIEW (Page, 1996). Only two of the six sequences encoded by the *A. niger* genome were included (Panneman et al., 1996, 1998). Human glucokinase was included for comparison.

transporters (Jørgensen et al., 2007). Expression of *mst*C is increased, and expression of *mstF* is reduced. Unlike the *N. crassa rco-3* gene, the *mst*A gene has been shown to encode a glucose transporter (see "cAMP" above) and does not contain a C-terminal extension. Thus, it is likely that the effects on *mst*C and *mstF* expression are due to the change in glucose uptake of the *A. niger mst*AΔ gene disruption mutant.

G-Protein-Coupled Receptors/cAMP Signaling

In *S. cerevisiae*, the G-protein-coupled receptor Gpr1p and the class III Gα subunit (Gα3) Gpa2p are involved in the increase of cAMP in response to glucose (see "Role of Hexokinases in Carbon Starvation" above; see also chapter 30). G-protein-coupled receptors and Gα subunits that are involved in glucose sensing have been identified in a number of filamentous fungi (reviewed by Li et al., 2007). There are some differences in these signaling pathways in filamentous fungi, but in many cases the role of these proteins is similar to their role in *S. cerevisiae* (Table 2).

In *N. crassa*, the G-protein-coupled receptor GPR-4 plays a role similar to that of yeast Gpr1p (Li and Borkovich, 2006). However, GPR-4 appears to be coupled to a Gα1 (GNA-1) rather than a Gα3 heterotrimeric G protein as in yeast. Furthermore, analysis of *N. crassa* mutants lacking Gα1 (GNA-1), Gα2 (GNA-2), or Gα3 (GNA-3) suggests that all three are involved in carbon sensing.

The *A. nidulans* and *Botrytis cinerea* Gα3 subunits have been shown to be involved in carbon-nutrient sensing and cAMP signaling in germinating conidia (Doehlemann et al., 2006; Lafon et al., 2005). In both species, compared to what is observed in yeast, a broader range of carbon sources, including glycerol and acetate, may be sensed by this signaling pathway. As in *A. nidulans* and *B. cinerea*, the Gα3 subunit of *Penicillium marneffei* is required for germination of conidia but it is not involved in nutrient sensing (Zuber et al., 2003). The Gα3 subunit of *Cryptococcus neoformans* controls cAMP production in response to glucose (Alspaugh et al., 1997). It also regulates melanin production in response to glucose starvation.

TABLE 3 Number of genes encoding hexokinases in filamentous fungi

Species	Phylum	No. of genes[a]	
		Ndt80-like	Hexokinases
C. cinereus	Basidiomycota	0	2
Cryptococcus neoformans	Basidiomycota	0	2
Phanerochaete chrysosporium	Basidiomycota	0	2
Puccinia graminis	Basidiomycota	0	5
U. maydis	Basidiomycota	1	2
C. immitis H538.4	Ascomycota	1	5
Batrachochytrium dendrobatidis	Chytridiomycota	2	2
A. nidulans	Ascomycota	2	5
C. immitis RS	Ascomycota	2	5
A. oryzae	Ascomycota	2	6
Aspergillus flavus	Ascomycota	2	7
Magnaporthe grisae	Ascomycota	3	4
N. crassa	Ascomycota	3	4
S. sclerotiorum	Ascomycota	3	4
A. niger	Ascomycota	3	5
B. cinerea	Ascomycota	3	5
Fusarium graminearum	Ascomycota	3	6
Aspergillus fumigatus	Ascomycota	3	8
Fusarium oxysporum	Ascomycota	4	7
Phycomyces blakeleeanus	Zygomycota	5	7
Rhizopus oryzae	Zygomycota	7	11

[a]The number of genes was determined by searching fungal genomes at the Broad Institute (http://www.broad.mit.edu/annotation/fgi/) and DOE Joint Genome Institute (http://genome.jgi-psf.org/).

GLUCOSE AS A REGULATORY MOLECULE

Many filamentous fungi have pathways for the metabolism of a wide range of potential carbon sources, and mechanisms that allow maximum energy efficiency for the organism have evolved. In most cases, expression of genes for dispensable carbon pathways is regulated by both pathway-specific induction controls and carbon catabolite repression. Carbon catabolite repression is a global regulatory phenomenon found in a wide range of microbial organisms and results in transcriptional repression of genes encoding less readily metabolized carbon sources in the presence of glucose, xylose, or sucrose, but not in the presence of arabinose, glycerol, melibiose, and lactose. Mannose, sorbitol, maltose, fructose, mannitol, and galactose lead to intermediate levels of repression (Arst and Cove, 1973).

Analysis of the regulation of metabolic pathways that are involved in the metabolism of carbon sources has been conducted using a range of techniques. Initially growth testing, enzyme assays, and mutant analyses were used to deduce pathways and regulatory mechanisms, and with the event of molecular biological techniques the components of the pathways were analyzed at the mRNA and protein level. With the availability of genomic data and the development of microarray methodologies, a whole-genome approach has been taken to the analysis of gene expression patterns between different growth conditions or genotypes. Advances in proteomic and metabolomic analysis techniques are extending the genome-wide analyses to provide an integrated understanding of the regulation of metabolic flux in the cell.

Studies of the regulation of pathways that provide a carbon source to the cell have been valuable in uncovering induction and carbon catabolite repression mechanisms, and these include studies of the regulation of quinate utilization in both *N. crassa* and *A. nidulans* (Bailey and Arst, 1975; Geever et al., 1989; Lamb et al., 1996; Levett et al., 2000) and ethanol utilization in *A. nidulans* (Arst and Bailey, 1977; Bailey and Arst, 1975; Flipphi et al., 2001; Kulmburg et al., 1992a, 1992b; Lockington et al., 1985; Nikolaev et al., 1999; Pateman et al., 1983; reviewed by Flipphi and Felenbok, 2004). Many compounds provide both a carbon and a nitrogen source to the cell, and studies of the regulation of these pathways are valuable in that they potentially provide an opportunity to unravel the interaction between carbon catabolite repression and ammonium repression. These include the study of the regulation of acetamidase (Hynes and Davis, 2004) and the enzymes for L-proline catabolism (Scazzocchio et al., 1995) in *A. nidulans*.

Mutations Leading to Carbon Catabolite Derepression

FrA Hexokinase and GlkA Glucokinase

Hexose-phosphorylating enzymes have been shown to be involved in glucose repression in *A. nidulans*, but unlike in yeast, different enzymes have overlapping roles. Mutations in *frA1*, which encodes a hexokinase, were selected due to their toxicity on D-fructose medium (McCullough et al., 1977), and mutations in *glkA*, which encodes a glucokinase, were selected due to their resistance to 2DOG in glycerol medium (Flipphi et al., 2003). Mutations in individual genes do not affect growth on glucose, nor do they lead to carbon catabolite derepression (Arst et al., 1990; Roberts, 1963; Ruijter et al., 1996); however, strains containing both *glkA* and *frA* mutations, which completely lack both glucose and

fructose phosphorylation, show derepression, indicating that GlkA and FrA activities compensate for each other in carbon catabolite repression signaling (Flipphi et al., 2003).

CreA Repressor Protein

The first genetic screen that aimed to isolate mutants with reduced carbon catabolite repression involved the selection of phenotypic suppressors of the effects of *areA* null alleles in *A. nidulans* (Arst and Cove, 1973). AreA is a DNA-binding protein that is required for expression of genes subject to ammonium repression (Arst and Cove, 1973; Caddick, 2004). The regulation of nitrogen metabolism is discussed in chapter 23, and thus, only brief comments to allow understanding of the selection rationale are presented here. Strains lacking AreA cannot grow on most nitrogen sources when glucose is present, but they can grow on compounds that provide both a carbon and a nitrogen source, such as acetamide and proline. Genes encoding enzymes for acetamide or proline metabolism are regulated by both carbon catabolite repression and ammonium repression, and the relief of either allows expression (Arst and MacDonald, 1975; Hynes, 1970). The observation that strains lacking AreA can grow when acetamide or proline are the only sources of both carbon and nitrogen, but not when glucose is also present, was the basis for the selection of mutations that allowed growth of *areA* null strains on glucose plus proline medium due to reduced carbon catabolite repression (Arst and Cove, 1973; Arst, 1981; Bailey and Arst, 1975; Hynes and Kelly, 1977). The *creA* gene was also identified in screens that involved selection of phenotypic suppressors of the requirement for an alternative source of acetyl-CoA due to *pycA* (pyruvate carboxylase) and *pdhA* (pyruvate dehydrogenase) mutant phenotypes (Arst and Bailey, 1977; Bailey and Arst, 1975; Romano and Kornberg, 1968, 1969). In addition, *creA* mutations were present among phenotypic suppressors of toxicity on fructose medium due to a *frA* (hexokinase) mutation (Arst et al., 1990; McCullough et al., 1977; Ruijter et al., 1996).

Mutations were also identified in other filamentous fungi. An industrial strain of *T. reesei*, Rut-C30, that overproduces cellulolytic enzymes, was found to contain a mutation in *cre1*, the *T. reesei* equivalent of *creA* (Ilmen et al., 1996). Mutations in *creA* have also been isolated in *A. niger*, using a screen involving *areA* null alleles similar to one that was used in *A. nidulans* (Ruijter et al., 1997).

Mutations in *A. nidulans creA* result in various degrees of deregulated expression of a wide range of genes that would normally be repressed in the presence of glucose, with no clear hierarchy for derepression (Arst and Cove, 1973; Arst, 1981; McCullough et al., 1977; Scazzocchio et al., 1995; Shroff et al., 1996, 1997). Both enzyme assay data and transcript analysis indicated that *creA* mutations also lead to elevated levels of gene expression under both carbon catabolite-repressing and derepressing conditions, indicating roles for CreA under growth conditions generally regarded as repressing as well as under derepressing conditions, although there are confounding experimental difficulties in identifying conditions that are completely nonrepressing without causing starvation and thus triggering a cellular starvation response.

In addition to studies in which individual genes or pathways have been analyzed, the transcriptional effects of a *creA* null mutation have been analyzed on a genome-wide scale using microarray analysis of *creA* wild-type and mutant strains grown on repressing and derepressing carbon sources (Mogensen et al., 2006). Cluster analysis was used to identify eight groups of genes based on their response, ranging from those that were unaffected to those that were greatly affected, although whether they were direct or indirect targets of CreA cannot be determined using this approach (Mogensen et al., 2006). This analysis validated and extended the data available from the analysis of individual pathways using Northern blots. Although a wide range of pathways show a degree of deregulated expression in *creA* mutant strains, some pathways are unaffected by the absence of *creA*, indicating that CreA-independent mechanisms of carbon catabolite repression exist. Examples include extracellular lipase (Kawasaki et al., 1995), penicillin biosynthetic enzymes (Espeso and Penalva, 1994; Martin et al., 1999) in *A. nidulans*, and D-xylulose kinase (VanKuyk et al., 2001) in *A. niger*. Mutations in *creA* also affect colony morphology (Arst et al., 1990; Shroff et al., 1997) and sensitivity to toxic compounds including acriflavine and molybdate.

The *A. nidulans creA* gene encodes a Cys_2-His_2 DNA-binding protein of the zinc finger class (Dowzer and Kelly, 1989, 1991). CreA homologues have been identified in a number of filamentous fungi, and as a result, conserved domains and regions can be identified (Shroff et al., 1997). The CreA proteins from filamentous fungi exhibit a high degree of sequence similarity (Fig. 4), and the zinc finger regions are highly similar to the zinc finger region of Mig1p, the carbon catabolite repressor in yeast, as is the C-terminal Mig1p effector domain (Ostling and Ronne, 1988; Ostling et al., 1996); however, other regions of the proteins from filamentous fungi show far less sequence similarity to Mig1p.

Mutations isolated in vivo fall into two clear classes; some are missense mutations in the zinc finger region of the protein which affect amino acids that are conserved among zinc finger proteins and are predicted to alter (*creA204*) or prevent (*creA306*) DNA binding, and others are nonsense or frameshift mutations that result in a truncated protein. Thus, the mutations that lead to derepression either alter the DNA binding region or result in the absence of the effector domain due to truncation (Shroff et al., 1996, 1997). An *A. nidulans creA* null mutant (constructed using gene replacement techniques) exhibited strong morphological changes and a high level of derepression (Shroff et al., 1997). However, the *creA306* mutation led to an even more extreme phenotype, perhaps due to titration of proteins that interact with CreA. The highly cellulolytic *T. reesei* strain (Rut-C30) is predicted to express a nonfunctional, severely truncated form of *T. reesei* Cre1 (Ilmen et al., 1996) and shows high levels of expression of various cellulase family mRNAs under repressing conditions.

CreA binds a DNA consensus recognition sequence, 5′ SYGGRG 3′, and this core recognition sequence is usually present in pairs (Cubero et al., 2000; Kulmburg et al., 1993; Mathieu et al., 2000; Panozzo et al., 1998). The same core recognition sequence is bound by Mig1p (Nehlin and Ronne, 1990; Nehlin et al., 1991; Papamichos-Chronakis et al., 2004). The presence of core sequences does not necessarily indicate functional binding in vivo, and the consensus sequences are present in many promoters that are not regulated by CreA. In addition, in many promoters, including those for *alcA* and *alcR*, only a subset of potential consensus binding sites are functional, indicating the importance of residues outside the core consensus sequence (Mathieu et al., 2000; Panozzo et al., 1998), or perhaps position effects.

Although the zinc finger regions of CreA and Mig1p are highly conserved, the mechanism of glucose repression in *S. cerevisiae* and *A. nidulans* is quite different. It was thought that Mig1p acts by recruiting the corepressor complex, Ssn6p-Tup1p, to promoters that are under carbon catabolite repression control (Nehlin et al., 1991; Treitel and Carlson, 1995; Tzamarias and Struhl, 1995) and that cytoplasmic

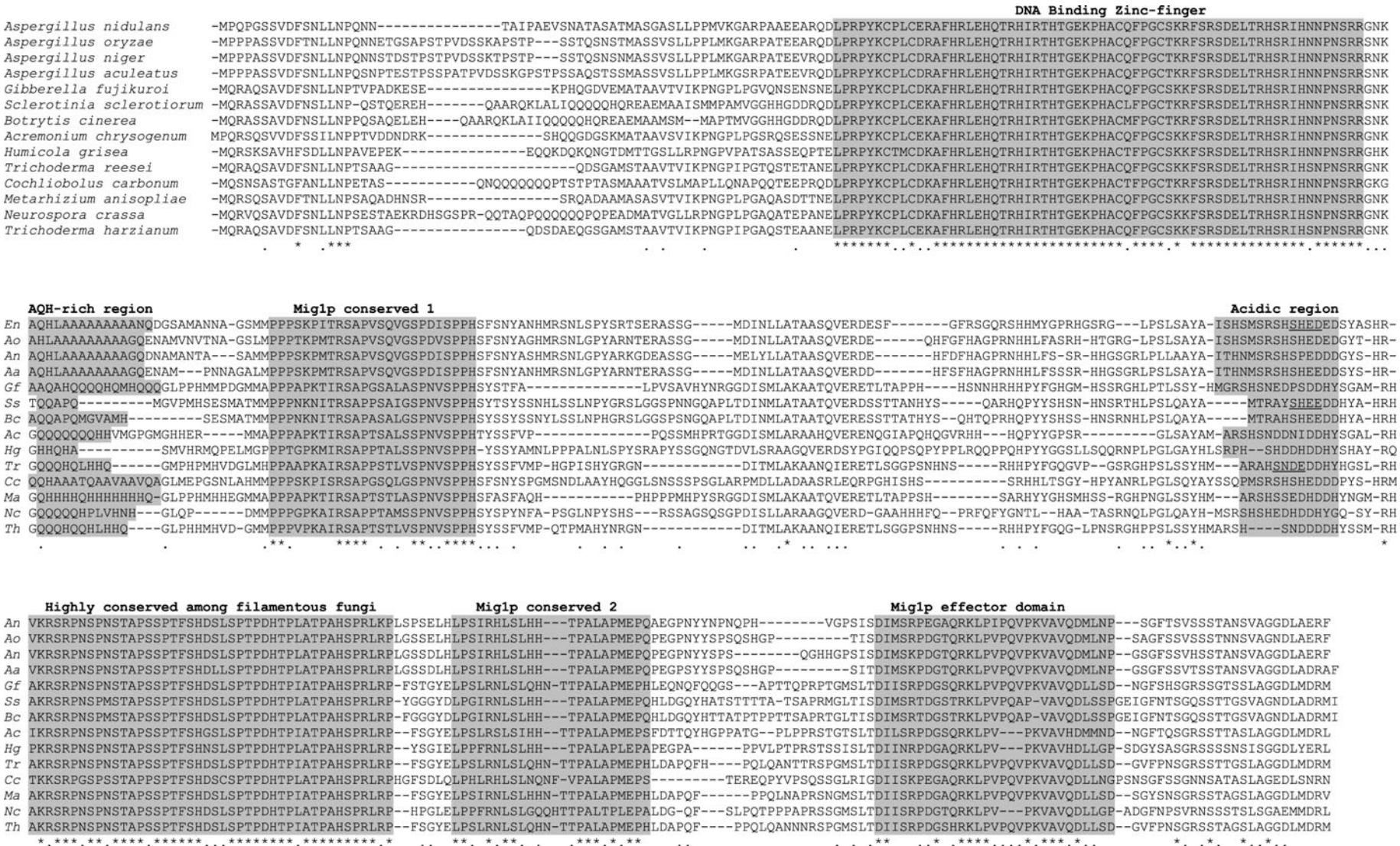
DNA Binding Zinc-finger
AQH-rich region
Mig1p conserved 1
Acidic region
Highly conserved among filamentous fungi
Mig1p conserved 2
Mig1p effector domain
Aspergillus nidulans
Aspergillus oryzae
Aspergillus niger
Aspergillus aculeatus
Gibberella fujikuroi
Sclerotinia sclerotiorum
Botrytis cinerea
Acremonium chrysogenum
Humicola grisea
Trichoderma reesei
Cochliobolus carbonum
Metarhizium anisopliae
Neurospora crassa
Trichoderma harzianum
En
Ao
An
Aa
Gf
Ss
Bc
Ac
Hg
Tr
Cc
Ma
Nc
Th

TABLE 4 Comparison of mechanisms of regulation by major carbon catabolite repression proteins in fungi

Mechanism or effect	Organism (gene/protein)			
	S. cerevisiae (*MIG1*/Mig1p)	*A. nidulans* (*creA*/CreA)	*T. reesei* (*cre1*/Cre1)	*S. sclerotiorum* (*CRE1*/CRE1)
Amount of transcript in repressed versus derepressed conditions[a]	–[b]	Lower	Lower	Higher
Regulated nuclear localization[c]	Yes	No	–	Yes
Regulated localization required for CCR[d,e]	No	No	–	No
Snf1-like protein[f] involved in CCR	Yes	No	No	–
Ssn6p/Tup1p complex[g] involved in CCR	Yes	No	–	–

[a]Amount of *creA*/*cre1*/*CRE1* mRNA in mycelia grown under carbon catabolite-repressed conditions compared to carbon catabolite-derepressed conditions.

[b]–, not tested.

[c]Whether the intracellular location of the protein is different in mycelia grown under carbon catabolite-repressed compared with carbon catabolite-derepressed conditions.

[d]CCR, carbon catabolite repression.

[e]Whether regulated nuclear localization is required for CCR, as determined by artificial disruption of export of the protein from the nucleus or by overexpression experiments.

[f]See "Hexokinase PII" and "CreA Repressor Protein" in the text.

[g]See "CreA Repressor Protein" and "CreB Deubiquitinating Enzyme and CreC SD40 Protein" in the text.

translocation of Mig1p, triggered by its phosphorylation by Snf1 kinase in the nucleus, was a key regulatory step for releasing glucose repression (De Vit and Johnston, 1999; De Vit et al., 1997). However, glucose still regulates Mig1p-dependent repression in a mutant that no longer has nuclear export activity, indicating that Mig1p is regulated both by nuclear localization and by modification of its ability to repress transcription (De Vit and Johnston, 1999). Further, the Ssn6p-Tup1p complex is tethered to the promoter DNA of *GAL1* under both repressing and activating conditions, and Mig1p is not required for the Ssn6p-Tup1p complex to be tethered to the *GAL1* promoter (Papamichos-Chronakis et al., 2002). Although Mig1p is not essential for the binding of the Ssn6p-Tup1p complex to the promoter, it is important for Ssn6p-Tup1p-mediated glucose repression (Papamichos-Chronakis et al., 2002). Snf1p-dependent phosphorylation of Mig1p abolishes the interaction with Ssn6p-Tup1p and controls transcriptional repression or derepression, and it is the regulation of this interaction, not the Mig1p cytoplasmic localization, that is the molecular switch that controls transcriptional repression and derepression of *GAL1* (Papamichos-Chronakis et al., 2002). Phenotypic analyses of strains lacking the *A. nidulans* Tup1p homologue, RcoA, have shown that deletion of *rcoA* does not greatly affect carbon catabolite repression (Hicks et al., 2001), and it is unlikely that CreA acts by recruiting RcoA as part of a general repressor complex.

Not only is carbon catabolite repression different between yeast and filamentous fungi, even among filamentous fungi the various CreA homologues appear to effect repression using different molecular methods (Table 4). In *A. nidulans*, *creA* transcription is autoregulated and *creA* mRNA is present in higher amounts in strains grown in relatively derepressing carbon sources such as arabinose than in strains grown in medium containing repressing levels of glucose (Arst et al., 1990; Shroff et al., 1996). However, a strain containing a transgene expressed from a constitutive promoter is phenotypically similar to the wild type, and thus, this autoregulation appears not to be a critical step in the regulatory mechanism (Roy et al., 2008). The *T. reesei* homologue *cre1* showed similar autoregulation of transcription (Ilmen et al., 1996). A rapid transient increase in *creA* transcript occurred on the addition of either glucose or a nonrepressing monosaccharide to carbon-starved mycelium, but these were down-regulated on repressing carbon sources (Strauss et al., 1999). However, in *Acremonium chrysogenum, cre1* showed glucose-dependent transcriptional upregulation, and this was absent from a strain of *A. chrysogenum* that displayed enhanced production of the β-lactam antibiotic cephalosporin C, consistent with the interpretation that the deregulation of *cre1* is connected with the increased production rate in this strain (Arst et al., 1990; Ilmen et al., 1996; Jekosch and Kuck, 2000a, 2000b; Shroff et al., 1996; Strauss et al., 1999). In *Sclerotinia sclerotiorum*, *cre1* transcript levels were also positively correlated with glucose concentration (Vautard-Mey et al., 1999).

The role of phosphorylation in the repressor activity of Cre1 has been investigated in *T. reesei* (Cziferszky et al., 2002, 2003). Phosphorylation of the Ser^{241} residue of *T. reesei* Cre1 was found to be required for DNA binding. Ser^{241}

FIGURE 4 Conserved motifs within filamentous fungal carbon catabolite repressor proteins. Shown are sequence comparisons of *En* (*Emericella* [*Aspergillus*] *nidulans* EMBL ENCREA); *Ao* (*A. oryzae* EMBL AOR272151); *An* (*A. niger* EMBL ANCREA); *Aa* (*Aspergillus aculeatus* EMBL AB024314); *Gf* (*Gibberella fujikuroi* EMBL GFY16626); *Ss* (*Sclerotinia sclerotiorum* EMBL SSCRES); *Bc* (*Botrytis cinerea* EMBL BCY16625); *Ac* (*Acremonium chrysogenum* EMBL ACH245727); *Hg* (*Humicola grisea* EMBL AB003106); *Tr* (*T. reesei* EMBL TR27356); *Cc* (*C. carbonum* EMBL AF306571); *Ma* (*Metarhizium anisopliae* EMBL MACRR1); *Nc* (*N. crassa* EMBL AF055464); and *Th* (*T. harzianum* EMBL THCRE1). Amino acid residues in *A. nidulans*, *S. sclerotiorum*, and *T. reesei* referred to in the text are underlined.

replaced by glutamic acid mimics phosphorylation, and Ser^{241} replaced by alanine allows binding without phosphorylation, and both these changes result in permanent carbon catabolite repression for cellobiohydrolyase I expression. On the other hand, a $Glu^{244}Val$ substitution leads to no phosphorylation, binding, or repression (Cziferszky et al., 2002). Snf1 kinase is not involved in phosphorylation of Cre1 in *T. reesei* (Cziferszky et al., 2002), and a casein kinase II target consensus surrounds Ser^{241}. However, in *S. sclerotiorum*, substitution of Ser^{266} in CRE1 (equivalent to Ser^{241} in *T. reesei*) by alanine leads to derepression (Vautard-Mey and Fevre, 2000). The *A. nidulans* CreA amino acid region $SHED^{262–265}$ corresponds to amino acids $SNDE^{241–244}$ in *T. reesei* and $SHEE^{266–269}$ in *S. sclerotiorum*. The observation that the *A. nidulans* strains containing deletions of this region are almost identical to the wild type with respect to repression and derepression indicates that this region is not essential for DNA binding or carbon catabolite repression in *A. nidulans* (Roy et al., 2008). In *Cochliobolus carbonum*, derepression of at least some carbon catabolite-repressible genes requires a Snf1p-like activity (Tonukari et al., 2000), but mutation of a *SNF1*-like gene in *A. nidulans* does not affect carbon repression (M. J. Hynes, personal communication).

By use of antibodies, CRE1 in *S. sclerotiorum* was detected at higher levels in glucose- and glycerol-grown mycelia than in pectin-grown mycelia, although there were no apparent differences in stability in the two media (Vautard-Mey et al., 1999; Vautard-Mey and Fevre, 2000). The subcellular localization of the protein varied with glucose concentration, and although the presence or absence of a potential serine phosphorylation site was correlated with repressor activity, it did not affect subcellular localization (Vautard-Mey et al., 1999; Vautard-Mey and Fevre, 2000). In *A. nidulans* strains expressing CreA:green fluorescent protein and exhibiting normal repression and derepression phenotypes, CreA:green fluorescent protein was present in the nucleus at high levels under both carbon catabolite-repressing and derepressing conditions. Thus, CreA activity is not regulated by subcellular localization in *A. nidulans* (Roy et al., 2008).

Strauss and colleagues showed that CreA was regulated at the posttranscriptional as well as the transcriptional level in *A. nidulans* (Strauss et al., 1999). They concluded that changes in activity of CreA could be due to covalent modification of the protein and/or protein degradation and noted the presence of a sequence similar to a consensus sequence involved in ubiquitination and proteasome-mediated degradation (Strauss et al., 1999). There is some evidence that at least a fraction of CreA in the cell is ubiquitinated (Kamlangdee, 2008), and Western blot analysis did not show significant differences in the total amount of CreA in cells grown under repressing or derepressing conditions, indicating that derepression does not require large-scale degradation of CreA (Kamlangdee, 2008).

SAGA complexes have been studied in yeast (reviewed by Baker and Grant, 2007), and they act through the coordination of multiple histone posttranslational modifications, including acetylation, methylation, ubiquitination, and phosphorylation. The Gcn5p subunit has histone acetyltransferase activity and acetylates histones. In *A. nidulans*, the relationship between transcriptional activation and nucleosome positioning has been investigated using the proline and ethanol regulons as a model (Garcia et al., 2004; Mathieu et al., 2005; Reyes-Dominguez et al., 2008). In the proline regulon, eight nucleosomes in the intergenic regulatory region are absent under inducing conditions, and they are partially restored under carbon- and nitrogen-repressing conditions when inducer is also present. CreA is required for partial nucleosome repositioning under repressing conditions, but not to establish the initial pattern. Trichostatin A, an inhibitor of deacetylases, results in total loss of nucleosome positioning under induced-repressing conditions (proline plus glucose), despite only a small derepression of *prnB*, indicating that CreA can repress independently of nucleosomes (Garcia et al., 2004). Transcriptional activation under derepressing conditions and chromatin remodeling were not affected in strains lacking GcnE and AdaB (essential components of the histone acetyltransferase), but the expression under induced-repressing conditions was affected along with the partial repositioning of histones under these conditions. This implies that nucleosome positioning, transcriptional activity, and histone acetylation are distinct rather than coupled processes and that GcnE and AdaB exert their function via CreA (Reyes-Dominguez et al., 2008).

CreB Deubiquitinating Enzyme and CreC WD40 Protein

Mutations in *creB* and *creC* were uncovered in some of the same screens that uncovered mutations in *creA*. Under glucose-repressing conditions, mutations in *creB* or *creC* result in a degree of deregulated expression of a subset of enzymes that would normally be subject to carbon catabolite repression. In the absence of repressing concentrations of glucose, these same alleles result in failure to express enzymes for the utilization of some carbon sources, such as quinate or proline (Arst, 1981; Hynes and Kelly, 1977; Kelly and Hynes, 1977). The effects of *creB* or *creC* mutations are pleiotropic, but there is no evidence of phenotypic heterogeneity among the alleles. Mutations in either gene increase sensitivity to acriflavine and decrease sensitivity to molybdate (Arst, 1981), and they have slight effects on colony morphology (Hynes and Kelly, 1977). *creB15* and *crec27* mutations greatly reduce uptake of proline or glutamate, but no effects could be measured on the uptake of glucose (Arst, 1981; Hynes and Kelly, 1977; Kelly, 1980). Biochemical studies have shown that mutations in *creB* and *creC* reduce external pH acidification, but the basis of this is not known, although it is known not to be due to defects in the plasma-membrane-bound H^+-ATPase (Abdallah et al., 2000; Espeso et al., 1995).

The *creB* gene encodes a ubiquitin-processing protease, upb, which is a member of the family defined by the human homologue UBH1 (Hansen-Hagge et al., 1998; Lockington and Kelly, 2001). The protein contains the six DUB (deubiquitination) homology domains common to this class of protein (D'Andrea and Pellman, 1998) and a carboxy-terminal extension (Lockington and Kelly, 2001). There are also high-scoring PEST sequences, which are usually implicated as signals for proteolysis and correlated with ubiquitination and rapid degradation (Rechsteiner and Rogers, 1996). The *creC* gene encodes a protein composed of a proline-rich region, a putative nuclear localization region, and five WD40 repeat motifs (Todd et al., 2000). Both CreB and CreC are conserved across eukaryotes. In *S. cerevisiae*, the Ssn6p-Tup1 general repressor complex is involved in carbon catabolite repression of genes regulated by glucose repression via the Mig1p DNA-binding protein, as well as in a number of other regulatory pathways (Treitel and Carlson, 1995; Tzamarias and Struhl, 1995). *S. cerevisiae* Tup1p shows weak sequence similarity to *A. nidulans* CreC within the WD40 repeat regions of each protein (Todd et al., 2000); however, Tup1p and CreC are not orthologous proteins, and *A. nidulans* RcoA is significantly more similar to Tup1p than is CreC (Hicks et al., 2001). Coimmunoprecipitation experiments

have shown that CreB and CreC are present in a high-molecular-weight complex in vivo, and each is not required for the presence of the other in the complex (R. A. Lockington, R. Murray, and J. M. Kelly, unpublished observations). CreB and CreC can be coimmunoprecipitated from mycelia grown under either carbon catabolite-repressing or carbon catabolite-derepressing conditions (Lockington and Kelly, 2002).

Some substrates of CreB have been identified. The quinate permease QutD is a ubiquitinated protein, and coimmunoprecipitation experiments have shown that it is a target of the CreB deubiquitinating enzyme (Kamlangdee, 2008). Further, levels of QutD are lower in a *creB* mutant strain than in a wild-type strain, indicating that deubiquitination by CreB is required to prevent protein turnover (Kamlangdee, 2008), and a failure to deubiquitinate ubiquitinated permeases is likely to be the underlying cause of the phenotypes of *creB* and *creC* mutations that are found under carbon-derepressing conditions. It is, however, not likely that these effects on permeases account for the all the derepression phenotypes due to these mutations, since the genes for ethanol utilization are partially derepressed in the mutants, and ethanol does not require a permease to enter the cell.

At least some of the pool of CreA, the carbon repressor protein, has also been found among proteins purified as ubiquitinated proteins; however, since there were no great differences in the total amount of CreA in cells grown under repressing or derepressing conditions, CreA activity is not regulated by wholesale degradation (Kamlangdee, 2008). Despite this, overexpression of CreA overcomes the defects in carbon catabolite repression seen in *creB* and *creC* mutant strains, and in coimmunoprecipitation experiments, a fraction of CreA precipitated with CreB, indicating that CreA is also a probable target of the CreB deubiquitinating enzyme (Kamlangdee, 2008).

Mutations Leading to Increased Carbon Catabolite Repression

CreD Arrestin Motif Protein

The *creC27* mutation results in hypersensitivity to fluoroacetamide in glucose medium due to derepression of acetamidase and acetyl-CoA synthetase, leading to the accumulation of toxic fluorocitrate. The *creD34* mutation was isolated as a suppressor of this hypersensitivity. *creD34* also suppresses some *creC27* phenotypes, such as derepression of alcohol dehydrogenase I, but not others, such as poor growth on quinate (Hynes and Kelly, 1977; Kelly, 1980). The *creD34* mutation also suppresses some of the phenotypic effects of the *creB15* mutation, and, weakly, of the *creA204* mutation (Hynes and Kelly, 1977; Kelly, 1980). In a wild-type background, the *creD34* mutation leads to increased resistance to acriflavine and reduced sensitivity to molybdate (the reverse of the phenotype due to *creA*, *creB*, and *creC* mutations) and to reduced utilization of some sugars and ω-amino acids. The CreD protein is highly similar to the Rod1p and Rog3p proteins from *S. cerevisiae*, and CreD was shown to interact with the ubiquitin ligase HulA, indicating that CreD is a component of a ubiquitination aspect of the glucose regulatory network that includes the CreB/CreC complex (Boase and Kelly, 2004).

AcrB

acrB mutant strains have a pleiotropic phenotype with respect to carbon source utilization. They show decreased ability to utilize a number of different sugars as sole carbon sources, including fructose, cellobiose, raffinose, and starch, in comparison to both the wild type and the *creD34* mutant strain, which may indicate a failure to derepress the enzymes required for their utilization (Boase et al., 2003). Mutations in *acrB* also lead to acriflavine resistance and sensitivity to molybdate (Boase et al., 2003; Boase and Kelly, 2004). The *acrB2* and *creD34* mutations also share other aspects of their mutant phenotypes, importantly suppression of aspects of the *creB15* and *creC27* mutant phenotypes such as allyl alcohol sensitivity. The effects on acriflavine resistance of the *acrB2* and *creD34* mutations are additive, and both genes encode proteins that, when mutated, can lead to tighter carbon catabolite repression (Boase and Kelly, 2004). That the *acrB2* mutation leads to suppression of the phenotypes due to the *creB* and *creC* mutations suggests that AcrB, like CreD, is involved in ubiquitination, since a failure to add ubiquitin to substrates could suppress the phenotypic effects of mutations that affect the removal of ubiquitin moieties. AcrB encodes a protein that contains three transmembrane domains and a coiled-coil region, with no highly similar proteins present in the genomes of yeast or higher eukaryotes (Boase et al., 2003). Thus, this represents a protein that performs a function that is either highly specific to filamentous fungi or is achieved by very divergent proteins in other organisms.

SUMMARY

There are significant differences in the mechanism(s) controlling gene expression in response to glucose in filamentous fungi and the well-characterized unicellular fungus *S. cerevisiae*. In addition, the few detailed studies of filamentous fungi that have been completed suggest that there are differences among these species as well. Similar DNA-binding proteins mediate carbon catabolite repression in filamentous fungi and *S. cerevisiae*. However, the regulation of the activity of this DNA-binding protein and its mechanism of action vary. Studies involving *A. nidulans* suggest that ubiquitination plays a role in carbon catabolite repression. The genes controlling this regulatory mechanism are not found in *S. cerevisiae*.

Little has been reported on the glucose-signaling mechanism that triggers changes in gene expression in filamentous fungi. Glucose phosphorylation has been shown to be necessary for glucose signaling in *A. nidulans*, but the role of hexokinases in glucose signaling in *A. nidulans* appears to differ from that of *S. cerevisiae*. A glucose transporter homologue (RCO-3), which may have a role in glucose signaling, has been identified in *N. crassa*, but the exact role of RCO-3 remains to be clarified. As in *S. cerevisiae*, G-protein-coupled receptors have been shown to have a role in sensing carbon nutrient status in filamentous fungi. The interaction of these signaling mechanisms has not been studied in filamentous fungi. Thus, an integrated picture of glucose signaling in filamentous fungi has yet to emerge.

Differences in the transport and metabolism of glucose also exist. *S. cerevisiae* possesses 17 closely related proteins that are capable of transporting glucose. Very few glucose transporters from filamentous fungi have been characterized. However, the transporters that have been identified appear to be more diverse in sequence. Further research will be required to determine how many different glucose transporters exist in filamentous fungi. The regulation of TCA cycle genes in response to glucose differs in *S. cerevisiae* and filamentous fungi, and as a consequence, the metabolism of glucose differs. In filamentous fungi, the expression of TCA

cycle genes is higher in glucose-rich medium and respiration rather than fermentation is favored. However, even among the few filamentous fungi that have been studied there are differences.

Given the central role of glucose in carbon metabolism and the diverse nutrient sources used by different fungi, it is not surprising that differences in glucose transport, glucose metabolism, glucose signaling, and carbon catabolite repression have arisen through selection. Most of the research on these pathways has been carried out with ascomycetes, and it is likely that additional differences will be discovered in other groups of fungi.

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Gluconeogenesis

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INTRODUCTION

Organisms require carbon substrates to generate energy and metabolites. The major pathways of carbon metabolism are glycolytic breakdown of sugars and the tricarboxylic acid (TCA) cycle for energy generation and the synthesis of biosynthetic intermediates. Filamentous fungi are able to use a very diverse range of compounds as carbon sources, consistent with growth in a variety of environments, including saprophytic growth on complex organic substrates, growth of pathogens in (or on) the host, and the use of storage compounds in reproductive spores. Growth on sugars requires glycolysis as well as the TCA cycle to generate energy and biosynthetic intermediates (chapter 21). Growth on carbon compounds metabolized via TCA cycle intermediates requires the net formation of sugars from TCA cycle intermediates in the process of gluconeogenesis—a reversal of glycolysis, in which TCA cycle intermediates are converted to sugars. This means that control of metabolism at the level of enzyme activity and enzyme synthesis in response to available carbon sources is absolutely vital to avoid futile cycling by glycolysis opposed by gluconeogenesis.

The availability of many annotated fungal genomes has revealed the complexities of carbon metabolic pathways and has highlighted our considerable ignorance of the function of many enzymes, their cellular localization, and the importance of the distribution of metabolites between cellular compartments. The impact of carbon metabolism on development and pathogenesis has been of increasing interest as fungal molecular genetics has developed. Furthermore, whole-genome analysis and attempts at systems biology approaches to metabolism afford opportunities for improvements in industrial uses of fungi.

A very thorough analysis of the state of knowledge regarding carbon metabolism in all filamentous fungi was published in 1986 (McCullough et al., 1986). This review now makes interesting reading in the light of the cloning and characterization of some of the genes discussed. In addition, the annotated genomes of fungi now available provide new information regarding the number of genes encoding isozymes, the possible cellular localization of enzymes, and the analysis of protein functions via molecular manipulations. For example, a particularly accurate and comprehensive annotation of relevant genes in *Aspergillus nidulans* has been published (David et al., 2006).

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Gluconeogenesis

Gluconeogenesis is summarized in Fig. 1. There are two enzymes that are essential for growth on gluconeogenic carbon sources. Phosphoenolpyruvate carboxykinase (PEPCK) (EC 4.1.1.32) converts the TCA cycle intermediate oxaloacetate to phosphoenolpyruvate, bypassing pyruvate produced by pyruvate kinase at the end point of glycolysis. Fructose-1,6-bisphosphatase (FBP) (EC 3.1.3.11), the final, irreversible step in hexose monophosphate formation, converts fructose 1,6-bisphosphate to fructose 6-phosphate, bypassing the glycolytic enzyme phosphofructokinase. The interconversion of fructose 6-phosphate to fructose-2, 6-bisphosphate is affected by ATP and phosphoenolpyruvate, with fructose 2,6-bisphosphate levels promoting glycolysis and inhibiting gluconeogenesis by allosteric regulation (Kubicek-Pranz et al., 1990; Mlakar and Legisa, 2006). FBP but not PEPCK is required for the generation of sugars from glycerol. The other enzymes required are reversible and shared with glycolysis. As described below, additional anaplerotic reactions may be required for the use of particular carbon sources. These replenish carbon intermediates used in biosynthetic pathways and ensure that the TCA cycle is functional. The best known of these is the glyoxylate bypass necessary for growth on substrates generating acetyl coenzyme A (acetyl-CoA). This pathway requires the unique enzymes isocitrate lyase (ICL) (EC 4.1.3.1) and malate synthase (MAS) (EC 4.1.3.2) as well as shared activities with the TCA cycle: citrate synthase, aconitase, and malate dehydrogenase (Kunze et al., 2006).

Importance for the Lifestyle of Fungi

Saccharomyces cerevisiae is specialized, with a strong preference for growth on fermentable monosaccharides generating ATP via glycolysis and not by respiration. However, when operation of the mitochondrial TCA cycle is compromised, key enzymes are turned on by the retrograde response to produce intermediates for biosynthetic reactions (Liu and

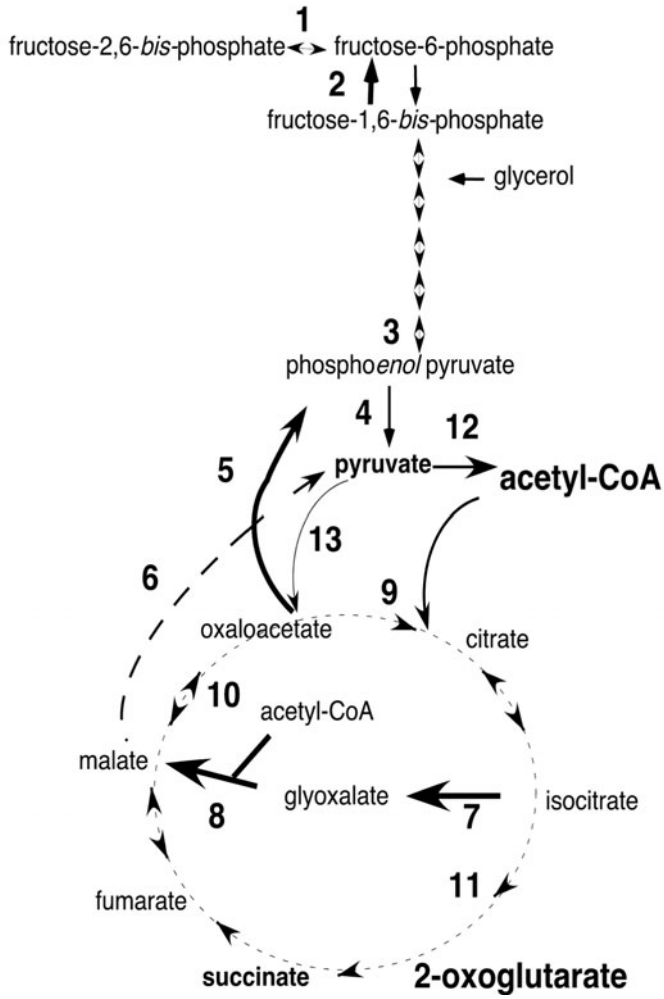

FIGURE 1 Outline of pathways for gluconeogenesis. Numbers in boldface represent key enzymes discussed in the text, as follows: 1, 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase; 2, fructose-1,6-bisphosphatase; 3, enolase; 4, pyruvate kinase; 5, phosphoenolpyruvate-carboxykinase; 6, malic enzyme; 7, isocitrate lyase; 8, malate synthase; 9, citrate synthase; 10, malate dehydrogenase; 11, isocitrate dehydrogenase; 12, pyruvate dehydrogenase; 13, pyruvate carboxylase. Reversible steps are shown with bidirectional arrows. For a full outline of relevant enzymes see Supplementary Figure S6 in David et al., 2006.

Butow, 1999). When glucose is exhausted, the metabolism switches to a respiratory mode in which the ethanol is consumed (Gancedo, 1998; Schuller, 2003). In contrast to filamentous fungi, *S. cerevisiae* can only use a limited number of substrates that result in the generation of TCA cycle intermediates as sole carbon sources (ethanol, acetate, and fatty acids), all of which result in the production of acetyl-CoA. Amino acids are not capable of acting as sole carbon sources but may be metabolized to fusel alcohols (Hazelwood et al., 2008). Some compounds are capable of acting as sole nitrogen sources but not as carbon sources, e.g., proline and γ-butyric acid (GABA).

In contrast, filamentous fungi are generally obligate aerobes, although *Neurospora crassa* is capable of considerable fermentation under high-glucose conditions (Xie et al., 2004) and under poor aerobic conditions fermentation is observed in *A. nidulans* (Lockington et al., 1997). Extremely slow growth on glucose, presumably via fermentation coupled with the alternative oxidase, has been observed in *A. nidulans* mutants lacking cytochrome *c* (Bradshaw et al., 2001). However, generally the TCA cycle is essential for growth on sugars and the retrograde response regulators of *S. cerevisiae* are apparently absent.

Consistent with growth on decaying plant material, fungi are capable of using a diverse range of gluconeogenic carbon sources, not just sources of acetyl-CoA but also amino acids and aromatic compounds (Hondmann and Visser, 1994; see also below). In the laboratory the ability to grow on particular carbon sources may be determined using defined media. In the real world, it is more likely that fungi grow on complex mixed carbon substrates with a consequent hierarchy of carbon utilization. It is also likely that mycelia undergoing carbon starvation in the wild are common, and survival depends on the breakdown of cellular components resulting in carbon sources requiring gluconeogenesis. Very little is known about the physiology of fungi under these circumstances. Of further relevance is fungal growth in fermenters with different growth-limiting parameters and substrates.

There has been increasing interest in the role of metabolism in processes such as spore formation and germination as well as pathogenesis. In asexual spores, mRNAs for gluconeogenic, glyoxylate cycle, and β-oxidation enzymes as well as peroxisomes are present, indicating that gluconeogenesis may be significant for spore survival and germination via the use of stored lipids (for examples, see Osherov and May, 2001; Osherov et al., 2002; Ebel et al., 2006; and Seong et al., 2008). Loss of citrate synthase and mutations affecting peroxisome function have been found to affect sexual development in *Podospora anserina* (Ruprich-Robert et al., 2002; Bonnet et al., 2006; see also chapters 15 and 33). Signaling by reactive oxygen species generated by NADPH oxidases is involved in both asexual and sexual reproduction in many fungi (Lara-Ortiz et al., 2003; Aguirre et al., 2005), and therefore the effects of growth on different carbon sources on NADPH levels are likely to alter development. In pathogenesis, infectious particles must germinate on host cell surfaces (often by the formation of specialized structures) or within host cells and initiate infection. Growth within host cells depends on available carbon substrates, and gluconeogenesis may be essential. Mutations affecting the glyoxylate cycle and fatty acid utilization can affect the pathogenicity and response to host cells of both plant and animal pathogens (Lorenz and Fink, 2002; Idnurm and Howlett, 2002; Solomon et al., 2004; Sexton and Howlett, 2006; Caracuel-Riosa and Talbot, 2007; Ibrahim-Granet et al., 2008). An example of particular relevance is afforded by infection by the rice blast pathogen *Magnaporthe grisea*, where turgor pressure required for leaf penetration by the appressorium depends on the conversion of lipids to glycerol via β-oxidation, the glyoxylate bypass, and gluconeogenesis (Wang et al., 2003, 2007; Caracuel-Riosa and Talbot, 2007). During infection by *Candida albicans* it has been shown that gluconeogenic enzymes are turned on (Barelle et al., 2006). Furthermore, the carbon substrate and the flux through central metabolic pathways affect the supply of essential intermediates for the production of signaling molecules such as oxylipins (Tsitsigiannis and Keller, 2007) as well as secondary metabolites such as antibiotics and mycotoxins (Maggio-Hall et al., 2005).

GLUCONEOGENIC CARBON SOURCES

Intermediary metabolites such as malate, pyruvate, and succinate are generally poor sole carbon sources, probably due to poor uptake. Other carbon sources metabolized to TCA cycle intermediates are excellent sole sources of carbon, and molecular genetic analysis of their mode of utilization has been performed for one or more filamentous ascomycete. These are summarized in Table 1. Using the *A. nidulans* sequence of a key indicator enzyme in a particular catabolic pathway for searches of the NCBI Uniprot database reveals that these pathways are conserved in filamentous ascomycetes (both pathogens and saprophytes) as well as in at least some basidiomycetes. The utilization of other weaker

TABLE 1 Strong gluconeogenic carbon sources common to filamentous ascomycetes

Carbon source	End product[a]	*A. nidulans* genes[b]	Enzyme[c]	Transcription factor(s)	Reference(s)
Acetate	Acetyl-CoA	*facA* AN5626	Acetyl-CoA synthetase	FacB	Todd et al., 1997, 1998
Ethanol	Acetyl-CoA	*alcA* AN8979	Alcohol dehydrogenase	AlcR	Flipphi and Felenbok, 2004
Fatty acids[d]	Acetyl-CoA	*foxA* AN7111	Multifunctional enzyme	FarA; FarB	Maggio-Hall and Keller, 2004; Hynes et al., 2006
Propionate	Pyruvate succinate	*mclA* AN8755	Methyl-isocitrate lyase	Not known	Brock et al., 2000
Benzoate[e]	Acetyl-CoA succinate	*bzuA* AN10950	Benzoate-4-monooxygenase	Not known	Fraser et al., 2002
Quinate[e]	Acetyl-CoA succinate	*qutB* AN1137	Quinate dehydrogenase	QutR; QutR	Giles et al., 1985; Grant et al., 1988
Glutamate	2-Oxoglutarate	*gdhB* AN7451	NAD-glutamate dehydrogenase	Not known	Arst et al., 1975; Kinghorn and Pateman, 1976
Proline	2-Oxoglutarate	*prnD* AN1731	Proline oxidase	PrnA	Gómez et al., 2002
GABA	2-Oxoglutarate succinate	*gatA* AN2248	GABA transaminase	AmdR	Arst, 1976; Richardson et al., 1989; Andrianopoulos and Hynes, 1990

[a]Intermediary metabolite(s) resulting from the specific catabolic pathway.

[b]*A. nidulans* genes used for blast-p searches on NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). AN numbers are from http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html.

[c]Enzyme activity chosen as key indicator of the presence of the specific pathway.

[d]Even-numbered fatty acids result in acetyl-CoA, while odd-numbered fatty acids result in both acetyl-CoA and propionyl-CoA, which is metabolized as for propionate.

[e]Benzoate and quinate are metabolized via the protocatechuate pathway (Hondmann and Visser, 1994). A detailed analysis of genomes for the quinate pathway has been presented (Hane et al., 2007).

carbon sources has also been studied, e.g., isoleucine and valine (Maggio-Hall et al., 2007) and leucine and aromatic amino acids (Peñalva, 2001).

Different TCA cycle end products are produced dependent on the carbon source (Table 1), and this has a major effect on the requirement for particular enzyme activities. Catabolism that generates only acetyl-CoA leads to a requirement for the glyoxylate cycle to produce C4 compounds. The production of 2-oxoglutarate alone results in the need to produce acetyl-CoA, which is required for biosynthetic pathways as well as citrate synthesis from oxaloacetate via citrate synthase activity enabling maintenance of the TCA cycle. This situation does not arise in *S. cerevisiae*, for which only sources of acetyl-CoA are gluconeogenic carbon sources.

Substrates Metabolized via Acetyl-CoA

Genetic and biochemical analyses of acetate utilization in *N. crassa* and *A. nidulans* were among the first performed to study carbon catabolism in fungi (Flavell and Fincham, 1968; Armitt et al., 1976). Subsequently, ethanol utilization via alcohol dehydrogenase and acetaldehyde dehydrogenase to yield acetate has been studied in detail (Flipphi and Felenbok, 2004). Threonine catabolism also yields acetate via acetaldehyde, but the specific threonine catabolic pathway has not been extensively studied. Acetate is converted to acetyl-CoA by acetyl-CoA synthetase in the cytoplasm and enters the mitochondrion as acetyl-carnitine produced by carnitine-acetyltransferase for metabolism via the TCA cycle and the peroxisome for metabolism via the glyoxylate cycle (see below). Mutations in the genes for each of these enzymes lead to loss of growth on acetate and ethanol but not on fatty acids (Armitt et al., 1976; Connerton et al., 1990; Stemple et al., 1998; Hynes et al., 2006). In contrast, mutations in the genes encoding the glyoxylate cycle enzymes ICL and MAS abolish growth on even-numbered-chain-length fatty acids as well as acetate and ethanol (Armitt et al., 1976; Hynes et al., 2006, 2008).

Filamentous ascomycetes can grow on a range of fatty acids, both long and short chain. In *S. cerevisiae*, a complete pathway for long-chain fatty acid β-oxidation, together with accessory enzymes, results in the production of acetyl-CoA in peroxisomes (Hiltunen et al., 2003). Multiple enzymes for steps in β-oxidation are found in filamentous fungal genomes. Many of these are predicted to be peroxisomal, and in some cases this has been shown (Bonnet et al., 2006; Maggio-Hall and Keller, 2004; Fosså et al., 1995). Deletion of orthologs of the genes encoding some of these enzymes has been found to affect fatty acid utilization (Maggio-Hall and Keller, 2004; Klose and Kronstad, 2006; Wang et al., 2007). However, it is apparent that there is a high level of redundancy, and a complete genetic elaboration of this pathway has not been accomplished for any filamentous fungus. In addition, mutants affected in peroxisome functions or proliferation exhibit phenotypic defects during growth on fatty acid media (Klose and Kronstad, 2006; Wang et al., 2007; Bonnet et al., 2006; Hynes et al., 2008). Unlike *S. cerevisiae*, filamentous fungi can use short-chain fatty acids, such as butyrate and hexanoate. A complete pathway for their conversion to acetyl-CoA in mitochondria has been discovered in *A. nidulans* (Maggio-Hall and Keller, 2004; Hynes et al., 2008). Using the sequence of the ScdA protein AN0824 (Maggio-Hall et al., 2007) to search fungal protein databases revealed that this pathway is likely to be conserved in filamentous fungi but not in hemiascomycetes, with the interesting exception of *Yarrowia lipolytica*. It is also clear that peroxisomes are required, and it has been suggested that there is a peroxisomal pathway for conversion of short-chain fatty acids to acetyl-CoA (Hynes et al., 2008). No mutants affected in this pathway have been discovered so far.

Substrates Metabolized via Propionyl-CoA

Propionate is a carbon source for fungi and has been shown to be converted to propionyl-CoA (Zhang et al., 2004). The metabolism of propionyl-CoA via the mitochondrial methyl-citrate cycle has been well studied in *A. nidulans* and *Aspergillus fumigatus*, and the relevant enzymes are conserved in other fungi (Brock, 2005; Brock and Buckel, 2004; Brock et al., 2000; Maerker et al., 2005; Ebel et al., 2006). Methyl-citrate synthase combines propionyl-CoA with oxaloacetate to form methylcitrate, which isomerizes to methylisocitrate, which is then cleaved to succinate and pyruvate. The production of these end products ensures that the glyoxylate cycle is not required.

The last step of β-oxidation of odd-numbered chain fatty acids will result in the production of propionyl-CoA as well as acetyl-CoA. In the case of valerate (C_5), a single round of β-oxidation will produce propionyl-CoA and acetyl-CoA. Valerate is a good carbon source for *A. nidulans* and does not require the defined mitochondrial short-chain pathway (Hynes et al., 2008). Propionyl-CoA has toxic effects due to inhibition of pyruvate dehydrogenase and succinyl-CoA synthetase (Brock and Buckel, 2004). This is particularly observed when this metabolite accumulates in methylcitrate synthase-deficient strains. In addition, polyketide secondary metabolite synthesis is inhibited (Brock, 2005).

Substrates Metabolized via Glutamate

Transamination of amino acids yields glutamate, and the corresponding keto acid and glutamate itself can serve as a sole carbon source. NAD-dependent glutamate dehydrogenase yields ammonium as well as 2-oxoglutarate. This enzyme is also essential for the use of amino acids metabolized via glutamate as either nitrogen or carbon sources (Arst et al., 1975; Kinghorn and Pateman, 1976). Release of ammonium is necessary to enable glutamine synthesis for nitrogen metabolism, while 2-oxoglutarate can enter the TCA cycle as a gluconeogenic carbon source. Consistent with this, PEPCK and FBP are required for the use of amino acids as carbon, but not nitrogen, sources in the presence of glycolytic carbon sources (Armitt et al., 1976; Hynes et al., 2007).

Proline, a very good sole carbon source for *A. nidulans*, is metabolized directly to glutamate. The genes for proline utilization (*prn*) are present in a cluster that includes *prnD* and *prnC*, encoding the two enzymes required for conversion of proline to glutamate; *prnX*, a gene of unknown function in proline utilization; *prnB*, specifying a proline permease; and *prnA*, encoding a pathway-specific activator (Gómez et al., 2002). A comparison with other fungal genomes at the Broad Institute website (http://www.broad.mit.edu/annotation/fungi/fgi/) indicates that the genes are conserved in other filamentous ascomycetes. However, there are two unlinked clusters in *Aspergillus oryzae* and *A. fumigatus*, and neither of these includes a permease gene. Clustering is not observed in *N. crassa* or *M. grisea*. The same pathway is found in *S. cerevisiae*, where the genes are scattered and are only involved in the use of proline as a nitrogen source (Des Etages et al., 2001).

The omega amino acid GABA is converted to 2-oxoglutarate and succinic-semialdehyde by a specific

transaminase required for growth on GABA as a carbon or nitrogen source (Richardson et al., 1989). Succinic-semialdehyde is metabolized to succinate by a dehydrogenase (Arst, 1976).

Aromatic Acids

The *qa* cluster of genes in *N. crassa* is required for growth on quinate, a product of lignin degradation, and has been the subject of classical studies in the laboratory of Case and Giles (Giles et al., 1985). A similar cluster has also been studied in *A. nidulans* (Grant et al., 1988), and related clusters are found in the genomes of other ascomycetes (Hane et al., 2007). A quinate permease, together with three enzymes, is specifically required for quinate utilization. The resulting protocatechuic acid, which is also a product of benzoate degradation, is then converted to succinate and acetyl-CoA by an additional seven steps encoded by dispersed genes (Hondmann and Visser, 1994).

CELLULAR LOCALIZATION OF METABOLITES AND ENZYMES

General Considerations

A critical problem in understanding carbon metabolism is the distribution of metabolites and enzymes between cellular compartments: the cytosol, mitochondrion, and peroxisomes. In general, both glycolysis and gluconeogenesis occur in the cytoplasm, while the TCA cycle is mitochondrial. Peroxisomes are required for fatty acid degradation and play a role (albeit ambiguous) in the glyoxylate cycle (see below). Peroxisomes have been generally defined as microbodies containing catalase, while glyoxysomes are microbodies lacking catalase but containing glyoxylate cycle enzymes. There is no clear case for distinguishing between the two classes (at least in fungi), as the same set of peroxisomal proteins (peroxins) are necessary for biogenesis and function. *N. crassa* peroxisomes lack catalase as well as other enzymes, such as acyl-CoA oxidase, generating reactive oxygen species, while *A. nidulans* has peroxisomal catalase and acyl-CoA oxidases (Schliebs et al., 2006; Kawasaki and Aguirre, 2001; K. Reiser and M. J. Hynes, unpublished data). It can also be noted that metabolites such as acetyl-CoA and methyl group donors must also be provided to nuclei for processes such as histone modifications, and this might be reflected in specific effects of growth substrates or mutations affecting primary metabolism on developmental stages. An indication of significant confusion in this area is illustrated by the different cellular localizations portrayed for the same reaction in metabolic diagrams in the literature. Our ignorance of the complexities of cellular localization poses significant difficulties for attempts to develop models and systems biology approaches to metabolism.

The classical approach to enzyme localization has involved cell fraction and enzyme assays. This has allowed the distribution of activities between the particulate (mitochondria) and soluble fractions (for an example, see Osmani and Scrutton, 1983). However, discrimination between isozymes (either resulting from different forms of the same gene product or the products of different genes) is not possible using this method. In addition, the biological significance of low-level activities in a fraction is not readily assessed. Annotated genomes now permit the isozyme complexity issue to be resolved, and cell biology approaches using tagged proteins allow a more accurate definition of cellular localization. However, detailed studies of each gene product, including analysis of the phenotypic effects of enzyme mislocalization, are required for a full understanding.

A further complicating factor is competition for the same substrate by enzymes in a compartment. In *S. cerevisiae*, TCA cycle enzymes form a complex in the mitochondrion and cytoplasmic malate dehydrogenase physically interacts with PEPCK and FBP, favoring flux through gluconeogenesis (McAlister-Henn and Small, 1997; Gibson and McAlister-Henn, 2003).

Distribution of Metabolites

The shuttling of metabolites between mitochondria, cytosol, and peroxisomes is crucial for gluconeogenesis. TCA cycle intermediates generated by specific catabolic pathways in the cytoplasm or peroxisomes must enter the mitochondria for further metabolism. Cytoplasmic PEPCK requires cytoplasmic oxaloacetate as a substrate. There are 34 members of the mitochondrial carrier superfamily responsible for the transport of metabolites through the mitochondrial membrane in *S. cerevisiae*, and many of these have been studied (Palmieri et al., 2000). Incredibly, the *A. nidulans* genome encodes 105 proteins annotated with this domain (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html), while *N. crassa* has 101 such proteins (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html), and some of these are predicted to be dicarboxylic carriers relevant to gluconeogenesis. Apart from the acyl-carnitine carrier (see below), the only known relevant predicted protein is the product of the *acuL* gene, originally identified as an *A. nidulans* acetate utilization mutant (Armitt et al., 1976). AcuL is an ortholog of the *S. cerevisiae* Acr1/Sfc1 succinate/fumarate transporter that is required for mitochondrial trafficking of succinate generated by the glyoxylate cycle. Consistent with this, AcuL is mitochondrial and required for growth on ethanol and fatty acids (M. J. Hynes, M. A. Davis, and R. B. Todd, unpublished observations).

The extent to which the single membrane of peroxisomes generally acts as a barrier to the flow of metabolites is not known (Tabak et al., 2008). No motifs that are signatures of carrier proteins involved in peroxisomal transport have been recognized. In *S. cerevisiae*, ABC half-transporters for fatty-acyl-CoA transport and acyl-CoA synthetase-coupled transport of free fatty acids are known (van Roermund et al., 2004). Furthermore, the ATP transporter Ant1 is proposed to be required for ATP import and maintenance of a proton gradient across the peroxisomal membrane (Palmieri et al., 2001; van Roermund et al., 2004). Mutations in a gene predicted to encode the orthologous protein in *A. nidulans* have been isolated; such mutant strains are unable to grow on fatty acids but able to grow on acetate (Hynes et al., 2008). There is the further relatively unexplored possibility of direct transport of metabolites between peroxisomes and mitochondria without passing through the cytoplasm.

The enzymatic generation of the appropriate reducing power in the form of NADH/NADPH in the different compartments is an important factor in the ability to use carbon sources. In *S. cerevisiae*, there are three genes encoding NADP-dependent isocitrate dehydrogenases, each of which is located in just one of the three compartments (Haselbeck and McAlister-Henn, 1993). In contrast, in *A. nidulans* a single gene encodes enzymes for each compartment, with the cytoplasmic enzyme probably being important for 2-oxoglutarate formation, while the mitochondrial and peroxisomal forms produce NADPH (Szewczyk et al., 2001). *S. cerevisiae* has a specific peroxisomal NAD-dependent

malate dehydrogenase for generating NADH (Kunze et al., 2002), while filamentous fungi do not have an equivalent enzyme with a peroxisomal targeting sequence.

Enzyme Localization in Mitochondria

The process of protein import into mitochondria has been well studied. There are established programs for the prediction of N-terminal mitochondrial targeting signals for proteins (for examples, see http://www.cbs.dtu.dk/services/TargetP/ and http://psort.ims.u-tokyo.ac.jp/), and the predicted TCA cycle enzymes of filamentous fungi contain these. However, these predictions are not absolute; for example, in *S. cerevisiae* fumarase is produced as a single protein targeted to both mitochondria and the cytoplasm (Sass et al., 2001; Regev-Rudzki et al., 2008). In *A. nidulans*, significant cytoplasmic activity is found (Osmani and Scrutton, 1983), and this is consistent with this enzyme converting fumarate to malate for gluconeogenesis in the cytoplasm. In other cases separate genes encode differentially localized isozymes. *S. cerevisiae* has three genes for NAD-malate dehydrogenase (Roth and Schüller, 2001), while in *A. nidulans* two genes are predicted to encode enzymes with no obvious targeting sequences in addition to the mitochondrial enzyme.

Enzyme Localization in Peroxisomes

Peroxisomal enzymes are localized in the matrix and are imported as folded proteins synthesized in the cytoplasm (Tabak et al., 2008). Import is carried out by peroxisomal membrane complexes comprising specific peroxins, the products of *pex* genes (chapter 15). In fungi there are two classes of peroxisomal matrix proteins: those containing a C-terminal PTS-1 sequence, which require the specific receptor Pex5 for import, and those containing an N-terminal PTS2 recognized by Pex7 (Brocard and Hartig, 2006; Petriv et al., 2004). Other peroxins such as Pex1, Pex6, and Pex13 are necessary for the import of both kinds of protein. Three C-terminal amino acids of the form S/A R/K L/M are the most common signatures of PTS1 proteins. However, there are variants of these, and the overall context of the 12 C-terminal amino acids can affect targeting (Brocard and Hartig, 2006). Conservation of C-terminal sequences in proteins from different species provides opportunities for assessing the likelihood of peroxisomal localization. In addition, cryptic internal PTS1 sequences have been found in proteins dependent on Pex5-mediated localization (for an example, see Klein et al., 2002). The PTS2 consensus (R/K L/V/I X5 H/Q L/A/F/I) close to the N terminus is found in the minority of proteins dependent on Pex7 for targeting (Petriv et al., 2004). An internal sequence conserved in the ICL of filamentous ascomycetes, but not in hemiascomycetes or plants (Gainey et al., 1992), has been found to be necessary for targeting in *A. nidulans*. The targeting is Pex7 dependent, thereby constituting a rare case of a cryptic PTS2 signal (Hynes et al., 2008).

Acetyl-CoA Metabolism and Peroxisomes

There are many aspects of the distribution of metabolites and enzymes required for growth on sources of acetyl-CoA that are poorly understood (for a detailed discussion see Kunze et al., 2006). Growth on acetate or ethanol results in the formation of acetyl-CoA in the cytoplasm via acetyl-CoA synthetase, while β-oxidation of fatty acids results in the synthesis of peroxisomal acetyl-CoA. Acetyl-CoA must then be metabolized via the TCA cycle in the mitochondrion and by the glyoxylate cycle.

It is clear that acetyl-CoA entry into mitochondria occurs as acetyl-carnitine formed by reversible carnitine acetyltransferase activities. A cytoplasmic enzyme (encoded by *facC* in *A. nidulans*) is required for growth on acetate, but not on fatty acids (Stemple et al., 1998). A second carnitine acetyltransferase (encoded by *acuJ* in *A. nidulans*) contains both mitochondrial and peroxisomal targeting (PTS1) sequences and is needed for growth on fatty acids as well as acetate (Stemple et al., 1998; Hynes et al., 2006; Bhambra et al., 2006; Ramos-Pamplona and Naqvi, 2006). An acylcarnitine carrier protein (encoded by *acuH* in *A. nidulans*) is required for transport of acetyl-carnitine into mitochondria, where it is converted to acetyl-CoA by carnitine acetyltransferase (De Lucas et al., 1999). It has been suggested that the peroxisomal membrane is impermeable to acetyl-CoA, but not to acetyl-carnitine (Elgersma et al., 1995; van Roermund et al., 1995, 1999; Swiegers et al., 2001). However, AcuH is mitochondrial, but not peroxisomal, and is required for growth on both fatty acids and acetate (De Lucas et al., 2001). This raises the possibility that acetyl-carnitine generated in peroxisomes by β-oxidation and carnitine acetyltransferase activity is directly transferred to mitochondria and not via the cytoplasm. We have recently shown that mislocalization of peroxisomal AcuJ to the cytoplasm by mutation of the PTS1 sequence results in an inability to grow on fatty acids without affecting growth on acetate (M. J. Hynes and S. L. Murray, unpublished data).

It should be noted that there are redundant pathways for the transfer of acetyl groups into mitochondria in *S. cerevisiae*: via acetyl-carnitine and via citrate formed by peroxisomal citrate synthase (Cit2) (Elgersma et al., 1995; van Roermund et al., 1995, 1999; Swiegers et al., 2001). The acetylcarnitine pathway is essential for growth on acetate and fatty acids in *C. albicans* as for filamentous ascomycetes (Zhou and Lorenz, 2008; Strijbis et al., 2008), while the citrate pathway is essential in plants (Pracharoenwattana et al., 2005).

The enzyme activities necessary for the glyoxylate cycle are distributed among mitochondria, cytoplasm, and peroxisomes. Citrate synthase and aconitase are required for the formation of isocitrate using acetyl-CoA and oxaloacetate produced from malate by malate dehydrogenase. Both citrate synthase and aconitase activities are primarily mitochondrial in *A. nidulans* (Osmani and Scrutton, 1983). In contrast, in *S. cerevisiae* there are both mitochondrial (Cit1) and peroxisomal (Cit2) citrate synthases and a minor, but significant, cytoplasmic aconitase activity (Regev-Rudzki et al., 2008). Interestingly, predicted citrate synthase enzymes in all *Aspergillus* spp. contain a PTS1 sequence as well as a mitochondrial targeting sequence. The significance of this is not clear. In *S. cerevisiae*, mitochondrial, peroxisomal, and cytoplasmically localized malate dehydrogenases are encoded by three separate genes (Roth and Schüller, 2001), while both cytoplasmic and mitochondrial isozymes are predicted in filamentous ascomycetes.

The unique glyoxylate cycle enzymes, ICL and MAS, are generally regarded as peroxisomal in fungi. MAS contains a PTS1 sequence. ICL from some, but not all, fungi, also possesses potential PTS1 sequences, while it seems likely that filamentous ascomycetes have a cryptic internal PTS2 sequence (Kunze et al., 2006; Hynes et al., 2008). However, in *S. cerevisiae*, ICL is cytoplasmic, while MAS, which has a PTS1, is peroxisomal in the presence of oleate but not acetate and mislocalization of MAS does not affect growth on oleate (Kunze et al., 2002). This indicates that peroxisomal localization is not essential. In filamentous fungi, peroxisomal localization of ICL and MAS is also not absolutely

required, because *pex* mutants affected in peroxisome biogenesis have been found to grow on acetate (Hynes et al., 2008). Furthermore, we have shown that mislocalization of MAS does not affect growth on either fatty acids or acetate (Hynes et al., 2008). This indicates the potential for the flow of metabolites between peroxisomes and the cytoplasm.

The specific situation for acetyl-CoA is of interest. It has been proposed that this can only exit or enter the peroxisome as acetyl-carnitine (Elgersma et al., 1995). However, the cytoplasmic carnitine acetyltransferase (FacC) is not required for growth of *A. nidulans* on fatty acids (Stemple et al., 1998; Hynes et al., 2006), and therefore, conversion of exported acetyl-carnitine to acetyl-CoA in the cytoplasm is not necessary. It is difficult to avoid the conclusion that acetyl-CoA produced by β-oxidation can exit the peroxisome for metabolism via the glyoxylate cycle and does not require conversion to acetyl-carnitine. However, import of acetyl groups into mitochondria is absolutely dependent on conversion of acetyl-CoA to acetyl-carnitine.

REGULATION OF GENE EXPRESSION

The regulation of genes required for growth on gluconeogenic carbon sources in response to the diauxic shift has been well studied in *S. cerevisiae*. However, the ability of filamentous fungi to grow on a wider range of gluconeogenic carbon sources means that different strategies for controlling gene expression are required. Although there are some related regulatory proteins involved in these control mechanisms, there appear to be fundamental differences among these groups of fungi.

Gluconeogenesis in *S. cerevisiae*

The extensive changes in gene expression accompanying growth on nonfermentable carbon sources are determined by the Snf1 master kinase that is activated when glucose levels are low (Gancedo, 1998). The resulting phosphorylation of the Cys2His2 zinc finger repressor protein Mig1 and the transcriptional activators Adr1, Cat8, and Sip4 turn on a large number of genes, including those for ethanol breakdown, the glyoxylate bypass, and gluconeogenesis (Schuller, 2003). Different genes are dependent to varying extents on the individual activators binding to carbon source response elements (CSREs) (Young et al., 2003; Tachibana et al., 2005). To add further complexity, yet another Snf1-dependent activator of gluconeogenesis, Rsd2, has recently been identified as binding to at least some CSRE elements (Soontorngun et al., 2007). Intriguingly, this protein is related to AcuM in *A. nidulans* and, furthermore, two other genes (YBR239C and YJL103C) specify proteins similar to AcuK (see below). In *S. cerevisiae*, all of the central genes required for gluconeogenic growth, including the glyoxylate cycle, are controlled transcriptionally by a lack of fermentable carbon sources rather than by induction by specific substrates. This is summarized in Fig 2.

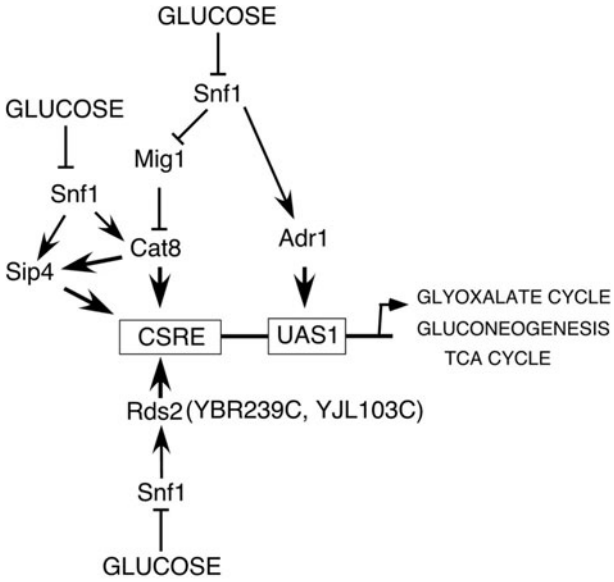

FIGURE 2 The logic of transcriptional regulation of gluconeogenic carbon source utilization in *S. cerevisiae*. The absence of glucose is the key signal. This results in the Snf1 kinase becoming active, leading to the removal of the repressor Mig1 from the nucleus and the activation of the transcriptional activators Cat8, Adr1, Sip4, and Rsd2 (and perhaps YBR239C and YJL103C) by phosphorylation. These activators regulate the transcription of a large number of genes involved in the utilization of carbon sources including both glyoxalate cycle and specific gluconeogenic genes. Each regulated gene is dependent to varying extents on two or more of the activators. The CSRE core element is CCAN5CCG, while the UAS1 consists of two half-sites, TTGGRG. In addition, Adr1, in conjunction with Oaf1 and Pip2, activates genes required for fatty acid utilization (Hiltunen et al., 2003). Based on data from Schuller (2003); Young et al. (2003); Tachibana et al. (2005); and Soontorngun et al. (2007).

Regulation in Filamentous Ascomycetes

Generally, specific catabolic pathways are controlled by pathway-specific induction using transcriptional activators—most commonly with Cys6 Zn binuclear cluster DNA binding domains. Examples of these are presented in Table 1, and the specific growth properties of loss-of-function mutations in these regulatory genes is shown in Table 2. Carbon catabolite repression of these pathways is mediated by conserved orthologs of the C2H2 Zn finger CreA repressor (Dowzer and Kelly, 1991; see also chapter 21). Commonly, repression of both structural genes (often permeases) and/or the pathway-specific transcription factor occurs. An example is provided by the ethanol system in *A. nidulans*, where both alcohol dehydrogenase (*alc*A) expression and *alcR* are subject to CreA-mediated repression (Flipphi and Felenbok, 2004). Therefore, specific pathways resulting in the formation of gluconeogenic substrates are expressed only if glucose is absent and inducer is present (Fig. 3).

There are fundamental differences with *S. cerevisiae*: deletion of genes for the Snf1 AMP kinase does not have pleiotropic effects on alternative carbon source utilization (for an example, see Yi et al., 2008), and obvious orthologs of Adr1 are absent from filamentous fungi. *A. nidulans* FacB and *N. crassa* Acu15, Zn(2)Cys(6) proteins, are related to Cat8 and Sip4 with similar DNA binding domains (Todd et al., 1997, 1998; Bibbins et al., 2002). However, in response to acetate, these proteins specifically regulate genes required for acetate utilization, i.e., acetyl-CoA synthetase, carnitine acetyltransferases, and the glyoxylate cycle enzymes ICL and MAS (Todd et al., 1998; Stemple et al., 1998; Bibbins et al., 2002; Hynes et al., 2006), and in contrast to Cat8 and Sip4, not those for gluconeogenesis. Mutations in *facA* and *facC* result in reduced FacB-mediated acetate induction, while an *acuJ* mutation enhances

TABLE 2 Carbon source utilization in loss-of-function regulatory gene mutants in *A. nidulans*[a]

Regulatory gene[b]	Carbon source						
	Glucose	Quinate	Ethanol	Acetate	Fatty acids	Proline	GABA
qutA	+	−	+	+	+	+	+
alcR	+	+	−	+	+	+	+
facB[c]	+	+	−	−	+	+	+
farA	+	+	+	+	−	+	+
prnA	+	+	+	+	+	−	+
amdR	+	+	+	+	+	+	−
acuK	+	−	−	−	−	−	−
acuM	+	−	−	−	−	−	−

[a]+, source is utilized; −, source is not utilized.

[b]See Table 1.

[c]Note that *facB* mutations affect growth on ethanol because ethanol is metabolized via acetate.

induction, indicating that acetyl-carnitine accumulation in the cytoplasm may be an important signal (Stemple et al., 1998). Mutations in two additional genes in *A. nidulans* specifically result in elevated levels of ICL in sucrose-grown cultures independently of FacB, but the function of these is not known (McCullough and Shanks, 1993).

In *S. cerevisiae*, fatty acid β-oxidation and peroxisome functions, but not ICL and MAS, are transcriptionally regulated by Oaf1 and Pip2 in response to long-chain fatty acids (Rottensteiner et al., 2003; Hiltunen et al., 2003). Completely different Zn(2)Cys(6) transcription factors, highly conserved in filamentous ascomycetes, not only transcriptionally control induction of these activities by fatty acids but also regulate other functions (e.g., the glyoxylate cycle enzymes and the peroxisomal/mitochondrial carnitine acetyltransferase) required for growth on both acetate and fatty acids separately from the specific acetate regulator, FacB (Hynes et al., 2006). FarA is required for induction by both short- and long-chain fatty acids, while FarB and ScfA are only necessary for short-chain induction. FarA and FarB orthologs were originally discovered as cutinase transcription factors involved in cutinase induction in *Nectria haematococca* (Li et al., 2002) and bind to a core CCGAGG sequence found upstream of many genes involved in the utilization of sources of fatty acids and in peroxisomal biogenesis and metabolism. FarA, but not FarB, orthologs are found in some hemiascomycetes such as *C. albicans* and *Y. lipolytica*, and CCGAGG sequences are present in the

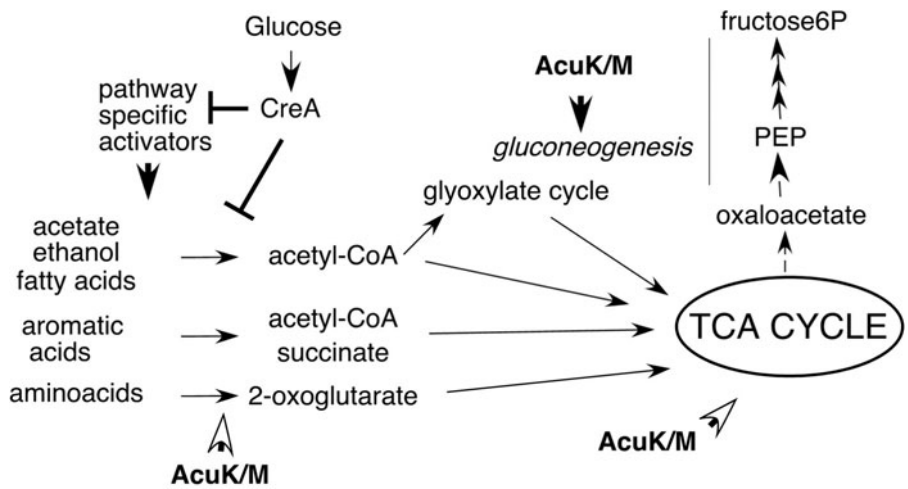

FIGURE 3 Proposed logic for transcriptional regulation of gluconeogenic carbon source utilization in *A. nidulans*. In the presence of glucose, the CreA repressor turns off the expression of the specific genes required for the utilization of each carbon source directly and/or by repression of the synthesis of a specific activator. However, this activator also requires an inducer for activity resulting in the genes for the specific pathway being turned on. Pathway-specific activators are given in Table 1. The specific pathways result in the production of TCA cycle intermediates. The AcuK/AcuM heterodimer is required for expression of the unique genes of gluconeogenesis as well as for at least some of the reversible glycolytic/gluconeogenic genes and is also proposed to increase the levels of at least some TCA cycle enzyme genes in response to the accumulation of TCA cycle intermediates. Black arrows indicate confirmed targets, while open arrows are proposed.

5′ end of relevant genes in these species (Hynes et al., 2006). This represents an extremely clear case of transcriptional rewiring within the Hemiascomycetes.

In filamentous ascomycetes, the genes for gluconeogenesis are regulated independently of the particular carbon source by a central control that reflects the buildup of TCA cycle intermediates and the requirement for hexose synthesis. Two key transcription factors required for this process have been identified in *A. nidulans* (Hynes et al., 2007). The *acuK* and *acuM* genes, which were originally identified as mutants unable to utilize acetate (Armitt et al., 1976), also affect growth on all carbon sources requiring gluconeogenesis (Table 2). Consistent with this, *acuK* and *acuM* mutations result in loss of induction of *acuF* (PEPCK) and *acuG* (FBP) by sources of TCA cycle intermediates such as acetate and proline. The *acuK* and *acuM* genes encode related transcription factors with similar Zn(2)Cys(6) DNA binding domains. Furthermore, database searches reveal that these genes are conserved in filamentous ascomycetes. Unexpectedly, we found that enolase (encoded by *acuN*), which is required for both gluconeogenesis and glycolysis, is expressed from two transcription starts: one controlled by glycolytic carbon sources and one by gluconeogenic carbon sources (Hynes et al., 2007). This raises the possibility that other genes encoding the reversible enzymes of glycolysis/gluconeogenesis are subject to dual control. This will not be observed in microarray experiments comparing glucose and gluconeogenic carbon source grown cultures (for an example, see David et al., 2006) if expression levels are similar on both glycolytic and gluconeogenic carbon sources. Detailed promoter analysis is necessary to resolve this for other glycolytic/gluconeogenic genes.

Recent work has identified binding sites for AcuK and AcuM. Electrophoretic mobility shift experiments with *Escherichia coli*-expressed maltose binding protein fusions have shown binding to DNA probes from the 5′ untranslated regions of *acuF*, *acuG*, and *acuN*, but only when both AcuK and AcuM proteins are present. Mutation of identified binding sites in an *acuF-lacZ* reporter has confirmed the in vivo significance of this binding. The minimal consensus sequence is a direct repeat, CCGN7CCG, and it appears that AcuK and AcuM bind cooperatively to activate expression (Y. Suzuki, M. A. Davis, and M. J. Hynes, unpublished observations). There is a striking conservation of both the sequence and position of these sites shown by in silico comparisons of the 5′ sequences of orthologous genes from other *Aspergillus* spp. The consensus site is also found in the 5′ untranslated regions of orthologous genes from more distantly related ascomycetes. The pattern of conserved 5′ sites also indicates the wide range of genes possibly subject to AcuK/M control. Upregulation of TCA cycle enzymes during growth on acetate was reported for *A. niger* and *A. nidulans* many years ago and in recent microarray experiments during growth on ethanol (Galbraith and Smith, 1969; McCullough et al., 1977; David et al., 2006). Genes for TCA cycle enzymes (e.g., orthologs of *S. cerevisiae SDH1*, *KGD1*, and *LPD1*), carboxylic acid mitochondrial transporters, NAD-dependent glutamate dehydrogenase and some glycolytic enzymes all have conserved sites in closely related species. It is clear that AcuK and AcuM play a central role in the rearrangement of metabolism during adaptation to growth on gluconeogenic carbon sources.

Intriguingly, the *N. crassa* orthologs (Aod2 and Aod5) of AcuK and AcuM have been found to regulate the alternative oxidase in response to antimycin inhibition of electron transport through complex III (Chae et al., 2007; see also chapter 13).These proteins have also been shown to bind cooperatively in vitro to CCGN7CCG sequences.

As in most Zn(2)Cys(6) proteins, the DNA-binding domains of AcuK and AcuM are N terminal, and conserved central domains (Pfam 04082) are present. Extensive stretches of amino acids at the C-terminal end of both proteins are conserved in the orthologs from other species. These contain similarities to PAS domains (pfam 00989) found in prokaryotic and eukaryotic proteins of diverse functions, including transcription factors, and act as sensors of various biological signals including light, oxygen, redox, and energy. Mutation of conserved residues in the PAS domains of the *N. crassa* Aod2 and Aod5 proteins eliminate induction of the alternative oxidase, thereby showing the importance of these domains (Chae et al., 2007). Interestingly the similar proteins found in *S. cerevisiae* (Rsd2 and the products of YBR239C and YJL103C; see above) also have C-terminal PAS domains. This raises the possibility that signaling to the transcription factors might reflect the required balance between TCA cycle flux and ATP generation by the electron transport chain. In addition, accumulation of TCA cycle intermediates results in induction of gluconeogenic gene expression in *A. nidulans*. The *acuF* mutation leads to increased expression of both *acuF-lacZ* and *acuG-lacZ* reporters and increased induction by malate, a weak source of induction in wild-type backgrounds (Hynes et al., 2007). This indicates that accumulation of oxaloacetate results in induction. However, oxaloacetate itself may not result in induction, since the reversible malate dehydrogenase and fumarase steps in the TCA cycle might lead to accumulation of fumarate. The nature of signaling to these gluconeogenic transcriptional activators remains to be determined. It is not clear why two transcription factors are needed, and it is possible that each protein responds to a different signal. Some genes might be regulated by homodimers of just of one of the proteins.

It is clear that AcuK and AcuM (and their orthologs in other fungi) play a major role in reprogramming metabolism in response to growth on gluconeogenic carbon sources. The full range of genes regulated needs to be addressed by microarray studies in wild-type and mutant backgrounds grown with different carbon sources. The relationship with additional control mechanisms operating during asexual and sexual development and in pathogenic infections is also of considerable interest.

SUMMARY

There is an increasing awareness of the complexities of carbon metabolism in fungi and of its importance for growth and development. The ability to use the sequenced genomes of many fungal species to identify and compare genes encoding metabolic enzymes provides many new opportunities for a fuller understanding. Furthermore, the availability of genome-wide approaches—microarrays, chromatin immunoprecipitation, proteomics, metabolomics, and systems biology—will allow a greatly increased expansion of knowledge. However, as discussed in this chapter, the complexities revealed by annotated genomes as well as recent studies should not be underestimated. These include (i) the occurrence of multiple genes encoding an enzyme activity; (ii) different proteins with different cellular localization signals encoded by a single gene; (iii) the possibilities for developmental and tissue-specific expression of pathways; (iv) the generation of substrates required for the production of signaling compounds and secondary metabolites; (v) the requirement for

counteracting metabolic stress (e.g., reactive oxygen species) during growth on particular substrates; and (vi) the situation in the real world, where mixed and limiting nutrients must be used, often in competition or cooperatively with other microbes, or where growth and development of pathogens on and in the host must occur. Clearly, detailed manipulations of individual genes, as well as whole-pathway analysis, are necessary.

It should also be noted that a comprehensive coverage of all aspects of the metabolism of gluconeogenic carbon utilization has not been attempted here. The role of many enzymes not discussed is ambiguous, and studies of the phenotypes of appropriate deletion mutants on a range of carbon sources, as well as of the effects of protein mislocalization, are necessary.

Despite the extensive analyses of carbon metabolism in *S. cerevisiae* that have been performed, there have been recent indications of unexplored complexities, as shown by the discovery of the role of the Rsd2 protein in transcriptional control (Soontorngun et al., 2007). The specialized physiology of *S. cerevisiae* is not always a good model for carbon metabolism in other fungi, not only in filamentous fungi but also in other hemiascomycetes. Therefore, an equivalent level of detailed analysis is needed in a wide range of fungi. Nevertheless, the discovery of the conserved AcuK and AcuM transcription factors as key regulators of the switch to growth on gluconeogenic carbon sources is a beginning to an understanding. In the filamentous ascomycetes, most is known about the laboratory models *A. nidulans* and *N. crassa*, the pathogens *M. grisea* and *A. fumigatus*, and the industrial species *A. niger* and *A. oryzae*. A broad attack on understanding metabolism in these organisms might be most rewarding. However, other organisms have provided particularly intriguing examples of specific relationships between metabolism and development, as exemplified by the work with *P. anserina* (chapters 15 and 33). Much less research on fundamental metabolism in other fungal phyla—the Zygomycetes and the Basidiomycetes—has been done, and these organisms have not been considered here. This is an important priority, as are investigations of mycorrhiza-plant metabolic interactions.

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Cellular and Molecular Biology of Filamentous Fungi
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Nitrogen Metabolism in Filamentous Fungi

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Nitrogen is an essential component of most biological macromolecules. The availability of nitrogen for assimilation into these compounds is therefore necessary for the growth, development, and reproduction of all living organisms. In the natural world, the fungi play a vital role in the recycling of nutrients through the degradation of organic material and the assimilation of environmental nitrogen. The metabolic versatility of the fungi is extraordinary, and their capacity to scavenge nitrogen is an important element in their success in occupying diverse environmental niches as free-living species and as opportunistic or obligate pathogens of plants and animals.

Among the filamentous fungi, the genetic basis of nitrogen metabolism has been most intensively studied in the model ascomycetes *Aspergillus nidulans* and *Neurospora crassa* by utilizing the excellent classical and molecular genetic systems provided by these species. Much of our current knowledge is based on classical genetic analysis of mutants affected in specific aspects of the enzymology or the regulation of nitrogen metabolism. These studies provided a foundation for the subsequent molecular genetic analysis (for reviews see Marzluf, 1981, 1997; and Caddick, 2004). Studies undertaken with other filamentous fungi reinforce many of the key aspects established in the model species. However, there are also instances where significant differences across species provide fascinating insights into the evolutionary divergence of nitrogen metabolism within the filamentous fungi. With the recent availability of multiple fungal genome sequences, it is now possible to look at the extent to which the fundamental processes of nitrogen metabolism and their underlying regulation are conserved. In this review, the molecular genetics of the ammonium assimilatory pathways is considered as the starting point for the biosynthesis of complex nitrogenous macromolecules. While ammonium or glutamine are the preferred nitrogen sources for fungi, their absence results in a need to synthesize catabolic enzymes in order to scavenge nitrogen from alternative sources. The switch from anabolism to catabolism requires the relief of nitrogen metabolite repression, a global control system that modulates the expression of large sets of nitrogen-catabolic enzymes. Recent studies suggest some diversity in the complex molecular mechanisms underlying this regulation among different fungal groups. Finally, we review the details of several nitrogen-catabolic systems to illustrate the metabolic and regulatory strategies employed by fungi in the acquisition of nitrogen metabolites.

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NITROGEN ASSIMILATION

Simple nitrogen-containing compounds released from the breakdown of more-complex substrates can provide sole sources of nitrogen for incorporation into new nitrogenous cellular components. The major route for the assimilation of ammonium in most fungi is via NADP-dependent glutamate dehydrogenase (NADP-GDH). This enzyme catalyzes the reductive amination of 2-oxoglutarate to produce glutamate and is a key step in linking the nitrogen and carbon cycles of the cell (Fig. 1). In *A. nidulans* and *N. crassa*, the NADP-GDH enzyme is encoded by the *gdhA* and *am* genes, respectively. Mutants lacking this activity are leaky glutamate auxotrophs and grow poorly when ammonium or sources of nitrogen assimilated via ammonium are the sole nitrogen source (Arst and MacDonald, 1973; Kinghorn and Pateman, 1975a; Kinnaird and Fincham, 1983). While animal GDHs can use either NAD or NADP as cofactors, fungal enzymes are usually NAD or NADP specific (Goldin and Frieden, 1971). In *A. nidulans* and *N. crassa*, a distinct NAD-linked GDH enzyme (encoded by the *gdhB* gene in *A. nidulans*) catalyzes the oxidative deamination of glutamate, forming 2-oxoglutarate and ammonium (Arst et al., 1975; Kinghorn and Pateman, 1976; Vierula and Kapoor, 1989). Unlike *gdhA* mutants, *gdhB* mutants are able to grow on ammonium as a sole nitrogen source but are unable to use glutamate or substrates metabolized via glutamate as sole carbon and nitrogen sources (Arst et al., 1975; Kinghorn and Pateman, 1976). The synthesis of these two GDH enzymes is regulated in an opposite fashion, consistent with their roles in either glutamate synthesis or catabolism. NAD-GDH activities are high in the presence of its substrate glutamate and under nitrogen or carbon limitation conditions, whereas NADP-GDH activities and *gdhA* transcript levels are high on ammonium and low in the presence of glutamate or under carbon-limited

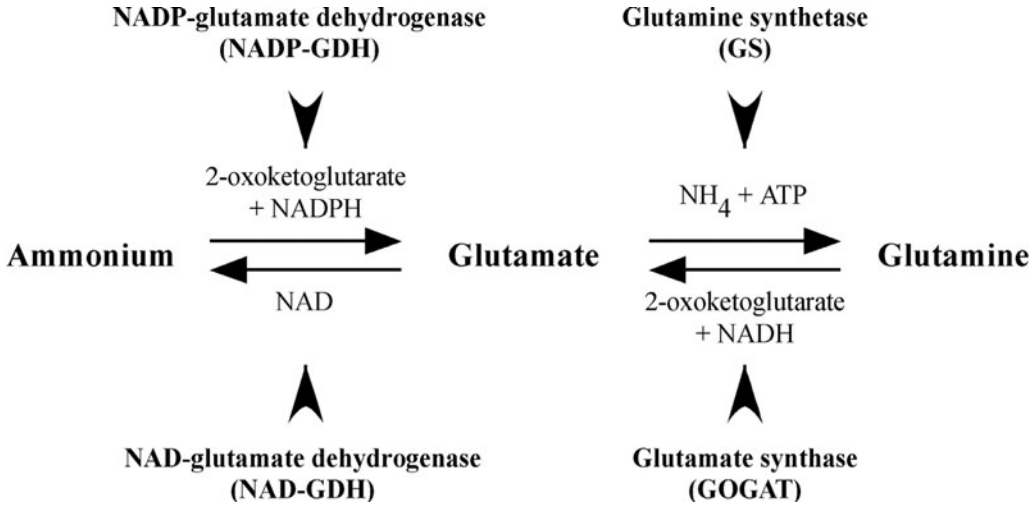


FIGURE 1 Ammonium assimilation pathway in fungi. Ammonium can be assimilated by NADP-GDH to form glutamate or by the GOGAT cycle through the action of GS and GOGAT to form glutamine and glutamate for the biosynthesis of nitrogenous molecules. The interconversion of ammonium, glutamate, and glutamine catalyzed by NADP-GDH, GS, GOGAT, and NAD-GDH is central to nitrogen metabolism.

conditions (Pateman, 1969; Kinghorn and Pateman, 1973, 1974; Hynes, 1974; Arst et al., 1975; Dantzig et al., 1979; Hernández et al., 1983; Hawkins et al., 1989).

Ammonium can also be assimilated by the GS-GOGAT cycle (Fig. 1) through the condensation of glutamate and ammonium to form glutamine catalyzed by glutamine synthetase (GS) and the conversion of glutamine and 2-oxoglutarate into two molecules of glutamate by glutamate synthase (GOGAT, NADPH-dependent glutamine:2-oxoglutarate amidotransferase). GS is encoded by the *gln*A gene in *A. nidulans* and the *gln-1* gene in *N. crassa*, while in *Fusarium (Gibberella) fujikuroi*, *gln*A-*Gf* is the GS structural gene (MacDonald, 1982; Cornwell and MacDonald, 1984; Margelis et al., 2001; Teichert et al., 2004). Mutants lacking this enzyme activity are unable to grow in the absence of glutamine, indicating that this is the sole route of glutamine biosynthesis. While GS mutants are strict glutamine auxotrophs, mutants of *A. nidulans* or *N. crassa* lacking GOGAT activity encoded by the *glt*A or *en-am* genes, respectively, do not have a discernible phenotype. The contribution of this enzyme to nitrogen assimilation is only apparent in double mutants lacking both NADP-GDH and GOGAT activities, where the residual capacity of *gdhA* or *am* mutants to grow on ammonium is abolished (Romero and Davila, 1986; Macheda et al., 1999). While NADP-GDH generally provides the major route of ammonium assimilation, in certain fungi the GS-GOGAT cycle is of more physiological importance (for an example, see Morel et al., 2006).

The molecular mechanisms controlling expression of the enzymes of nitrogen assimilation have not been well defined. Studies of NADP-GDH expression thus far suggest that multiple regulatory proteins modulate *A. nidulans gdhA* and *N. crassa am* gene expression. Dissection of the *am* gene promoter revealed two functionally important upstream regions, designated US*am*α and US*am*β (Frederick and Kinsey, 1990). The US*am*α site was shown to bind a heteromeric complex, AAB, homologous to the HAP CCAAT binding complex of *Saccharomyces cerevisiae* (Chen and Kinsey, 1994; Chen et al., 1998). In *A. nidulans*, high-level expression of *gdh*A also requires the activity of the CCAAT binding complex encoded by the *hapB*, *hap*C, and *hapE* genes (Steidel et al, 1999). In addition, regulatory proteins encoded by *A. nidulans* and *Aspergillus oryzae are*A and the *N. crassa nit-2* genes are required for wild-type levels of NADP-GDH activity on ammonium (Dantzig et al., 1979; Calderón and Martínez, 1993; Christensen et al., 1998). AreA and Nit2 are GATA transcription factors and function as global regulators of nitrogen metabolite repression (see "Global Regulation of Nitrogen Catabolism" below). The role of these transcription factors in activating NADP-GDH synthesis on ammonium is interesting. AreA and Nit2 activate nitrogen-catabolic gene expression under conditions of nitrogen limitation, and neither protein is active at catabolic gene promoters under nitrogen-sufficient conditions. However, they clearly have a role activating NADP-GDH synthesis in cells grown on ammonium as the sole nitrogen source. At least in the context of the *gdhA* promoter, the activity of AreA may depend on interaction with two $Zn(II)_2Cys_6$ transcription factors: TamA, an AreA coactivator, and LeuB, the *A. nidulans* homologue of *S. cerevisiae* Leu3 (Davis et al., 1996; Small et al., 2001; Polotnianka et al., 2004). The phenotypes of *tamA* and *leuB* loss-of-function mutants indicate that both gene products play a significant role in *gdhA* expression in *A. nidulans*, and *tamA* mutations have been associated with altered ammonium assimilation (Kinghorn and Pateman, 1975b, 1976; Arst et al., 1982; Davis et al., 1996; Polotnianka et al., 2004). TamA has been shown to interact with AreA and LeuB by yeast two-hybrid analysis, and TamA can recruit AreA to promoters when fused to a heterologous DNA binding domain (Small et al., 1999; Polotnianka et al., 2004). These studies suggest that all three proteins may function in a complex at the *gdhA* promoter. LeuB has an additional role in the regulation of leucine biosynthesis and *leuBΔ* mutants are leaky leucine auxotrophs (Polotnianka et al., 2004). *S. cerevisiae* Leu3 regulates *GDH1*, encoding NADP-GDH, and the activity of Leu3 is modulated by levels of α-isopropylmalate, an intermediate in leucine biosynthesis

(Sze et al., 1992; Hu et al., 1995). Similar regulation of LeuB activity would provide a mechanism to adjust glutamate synthesis via NADP-GDH in response to intracellular amino acid levels during growth on ammonium.

GLOBAL REGULATION OF NITROGEN CATABOLISM

When provided with a choice of nitrogenous substrates, most fungi will preferentially metabolize ammonium and glutamine. However, in the absence of these readily assimilated nitrogen sources, filamentous fungi can reclaim nitrogen from a wide range of alternative sources in their environment through the synthesis of various nitrogen-catabolic enzymes. The synthesis of these enzymes is regulated by a global regulatory control mechanism—nitrogen metabolite repression—that modulates the expression of catabolic genes in response to nitrogen availability. In addition, the expression of genes in certain catabolic pathways requires induction by their substrates mediated by pathway-specific transcriptional activators (for examples, see Andrianopoulos and Hynes, 1990; Burger et al., 1991a, 1991b; Fu et al., 1995; Suarez et al., 1995; Gómez et al., 2002). The nature of the signal(s) of nitrogen sufficiency is not established, but studies of *A. nidulans* and *N. crassa* using mutants lacking NADP-GDH or GS activity indicate that endogenous levels of glutamine or its derivatives are used as signaling molecules to monitor the nitrogen status in the cell (Kinghorn and Pateman, 1973; Dunn-Coleman and Garrett, 1980; Dantzig et al., 1978; Margelis et al., 2001).

The major fungal nitrogen regulatory protein is a GATA transcription factor required to promote expression of nitrogen-catabolic genes when nitrogen is limiting. These proteins contain a single C-terminal $CX_2CX_{17}CX_2C$ DNA binding domain related to the mammalian GATA zinc finger proteins that bind a core 5′-GATA-3′ DNA sequence (Fu and Marzluf, 1990a, 1990b; Kudla et al., 1990; see also Scazzocchio, 2000). In *A. nidulans* and *N. crassa*, the global nitrogen regulator is encoded by the *areA* and *nit-2* genes, respectively, and loss-of-function mutants are unable to use nitrogen sources other than ammonium or glutamine (Marzluf, 1997) (see Fig. 4A). The functional conservation of these genes was initially demonstrated by the complementation of an *areA* mutant using the *N. crassa nit-2* gene (Davis and Hynes, 1987). Homologous genes have been identified in various *Aspergillus* and *Penicillium* species, as well as a number of fungal pathogens by the characterization of loss-of-function mutants and/or heterologous complementation of either *areA* or *nit2* mutants (Haas et al., 1995; Froelinger and Carpenter, 1996; Christensen et al., 1998; Hensel et al., 1998; MacCabe et al., 1998; Screen et al., 1998; Gente et al., 1999; Tudzynski et al., 1999; Chang et al., 2000; Perez-Garcia et al., 2001; Pellier et al., 2003; Yamada et al., 2006). AreA homologues are highly conserved within the *Aspergillus* species and their close relatives, whereas NIT-2 and its homologues in species more closely related to *N. crassa* are diverged from AreA, consistent with the phylogenetic relationship between these species (Table 1). However, alignment of these fungal homologues reveals that there are several short conserved sequences throughout the protein, in addition to the extended amino acid conservation in the region of the GATA DNA binding domain, where AreA and NIT-2 differ by a single conservative amino acid substitution (Fu and Marzluf, 1987; Kudla et al., 1990; Wong et al., 2008).

Signals generated during growth under nitrogen-sufficient conditions serve to prevent these GATA factors from activating catabolic gene expression. How the activity of these regulatory proteins is modulated under different conditions remains an important question. While in *S. cerevisiae* the target of rapamycin cascade regulates the response to nitrogen status of the cells, the target of rapamycin kinase appears to play a more minor role in nitrogen regulation in filamentous fungi (Fitzgibbon et al., 2005; Teichert et al., 2006; Schönig et al., 2008). Both transcriptional and posttranscriptional controls have been shown to influence the levels and activity of AreA in response to nitrogen availability (Fig. 2). The last nine residues at the extreme C terminus of AreA and NIT-2 are absolutely conserved, and similar conserved regions are identified in the comparison of fungal AreA homologues. Detailed studies in *N. crassa* showed that these residues, together with residues in the NIT-2 GATA finger, interact with the negatively acting protein NMR (Dunn-Coleman et al., 1981; Fu et al., 1988; Young et al., 1990; Xiao et al., 1995; Pan et al., 1997). Loss-of-function *nmr* mutations result in derepression of nitrogen-catabolic gene expression, and deletion of the equivalent *A. nidulans nmrA* gene or C-terminal truncation of AreA also leads to a partial insensitivity to repression (Debusk and Ogilvie, 1984; Jarai and Marzluf, 1990; Stankovich et al., 1993; Platt et al., 1996a, 1996b; Andrianopoulos et al., 1998). It is not known whether the interaction with NmrA inhibits AreA activation and/or DNA binding under nitrogen-sufficient conditions. However, structural studies indicate that binding of the AreA zinc finger to GATA sequences and to NmrA is mutually exclusive, suggesting that NmrA may function to inhibit AreA DNA binding (Kotaka et al., 2008). NmrA contains a Rossmann fold identified in other corepressor proteins and preferentially binds oxidized rather than reduced nicotinamide dinucleotides (Stammers et al., 2001; Zhang et al., 2002; Lamb et al., 2003, 2004; Thio et al., 2004; Thoden et al., 2007). While nucleotide binding to NmrA may have a redox-sensing function, recent analysis of the crystal structure of the AreA zinc finger complexed with NmrA indicated that the conformation of the complex was not altered in the presence or absence of $NAD(P)^+$ (Kotaka et al., 2008). In vivo studies have shown that overexpression of *nmrA* is sufficient to inhibit AreA activity, even under conditions of complete nitrogen starvation (Wong et al., 2007). Thus, the AreA-NmrA interaction does not require a signal generated during nitrogen sufficiency, highlighting the levels of NmrA relative to AreA as the critical factor. NmrA levels are elevated under nitrogen-sufficient compared with nitrogen starvation conditions, and this is achieved through regulated expression of *nmrA* by the bZIP transcription factor MeaB (Polley and Caddick, 1996; Lamb et al., 1997; Wong et al., 2007). Therefore, the activity of MeaB may be the determining factor in the control of *nmrA* expression and the signals of nitrogen sufficiency may trigger changes in MeaB conformation and/or dimerization that ultimately regulate AreA activity.

Homologues of NmrA and MeaB are unique to the filamentous ascomycete fungi. Comparison of the overall similarity of the NmrA homologues reveals an extremely high level of amino acid conservation in the Eurotiomycetes (Table 1). While this comparison suggests that NmrA homologues in the Sordariomycetes and Leotiomycetes have significantly diverged from NmrA, direct alignment of the predicted proteins shows that these contain highly conserved 'core' NmrA sequences, including the NAD-binding domain flanked by nonconserved N-terminal and C-terminal

TABLE 1 Conservation of nitrogen regulators in filamentous ascomycetes[a]

Species	AreA		NmrA		MeaB	
	% Identity	% Similarity	% Identity	% Similarity	% Identity	% Similarity
Eurotiomycetes						
A. flavus	69.4	77.6	88.1	94.3	76.8	85.4
A. oryzae	69.5	77.7	87.8	94.0	76.8	85.4
A. terreus	65.9	73.6	86.9	93.5	77.9	85.9
A. niger	70.3	78.8	88.9	94.6	76.5	84.7
N. fischeri	69.8	78.5	88.1	94.3	73.3	82.1
A. fumigatus	70.2	79.4	87.5	94.9	73.8	83.1
A. clavatus	68.8	77.6	86.9	93.8	70.8	82.6
C. immitis	41.4*	50.0*	71.8	83.4	46.8	59.9
U. reesii	36.9	45.6	69.3	82.3	47.1	62.1
H. capsulatum	29.8*	40.9*	58.9*	69.6*	45.9	56.9
Sordariomycetes						
M. grisea	35.8	48.3	36.9	46.0	39.0	50.4
N. crassa	35.1	46.4	37.5	46.0	38.3	49.0
C. globosum	36.1	47.3	39.1	47.1	40.6	52.4
T. reesei	36.9	48.3	41.4	49.4	39.4	50.2
F. graminearum	37.4	48.0	41.7	48.7	41.5	50.4
F. oxysporum	35.3	45.4	42.1	49.6	41.9	53.6
F. verticillioides	35.4	45.7	39.9	46.6	41.7	53.6
Leotiomycetes						
B. cinerea	40.7	52.6	38.8	48.3	43.8	56.7
S. sclerotiorum	39.0	48.8	38.8	48.2	42.4	56.0

[a]The protein sequences of AreA, NmrA, and MeaB orthologues from fungi belonging to Eurotiomycetes, Sordariomycetes, and Leotiomycetes within the Ascomycota lineage (Fitzpatrick et al., 2006) are identified by blastp and tblastn searches (Altschul et al., 1997) using the respective *A. nidulans* sequence from the respective fungal genome sequence database in Broad Institute (http://www.broad.mit.edu/annotation/fgi/) and DOE Joint Genome Institute (http://genome.jgi-psf.org/Trire2/Trire2.home.html). The orthologous AreA, NmrA, and MeaB sequences from various fungi are compared to the respective orthologue in *A. nidulans*, using the Needle program in Biomanager, Australian National Genome Information Service (ANGIS, http://www.angis.org.au). Accession numbers are listed below when possible; otherwise, the gene locus (GL) number in the respective genome sequence database is indicated. Accession numbers or gene locus numbers for AreA are as follows: *A. flavus* (GL, AFL2G_10206.2); *A. oryzae* (O13415); *A. terreus* (EAU32648); *A. niger* (CAA68196); *N. fischeri* (EAW15479); *A. fumigatus* (EAL85842); *A. clavatus* (EAW08915); *C. immitis* (EAS27400); *U. reesii* (GL, UREG_06018.1); *H. capsulatum* (GL, HCAG_05031.1); *M. grisea* (Q01168); *N. crassa* (P19212); *C. globosum* (EAQ89860); *T. reesei* (GL, 76817); *F. graminearum* (GL, FGSG_08634.3); *F. oxysporum* (GL, FOXG_03165.2); *F. verticillioides* (GL, FVEG_02033.3); *B. cinerea* (GL, BC1G_09852.1); and *S. sclerotiorum* (GL, SS1G_05040.1). See supplementary materials from Wong et al., 2007, for accession numbers of NmrA and MeaB orthologues, except for those of *C. immitis* (NmrA, EAS31008; MeaB, GL, CIMG_03780.2); *U. reesii* (NmrA, GL, UREG_03933.1; MeaB, GL, UREG_07503.1); *H. capsulatum* (NmrA, EDN02584; MeaB, GL, HCAG_01916.1); *T. reesei* (NmrA, GL, 74375; MeaB, GL, 73417); *F. oxysporum* (NmrA, GL, FOXG_01593.2; MeaB, GL, FOXG_02277.2); and *F. verticillioides* (NmrA, GL, FVEG_07953.3; MeaB, GL, FVEG_05452.3). Asterisks (*) indicate sequences that contain potential annotation errors. The EAS27400; GL, HCAG_05031.1; CIMG_03780.2; and HCAG_01916.1 sequences have been manually reannotated.

extensions (Wong et al., 2007). It appears that the acquisition (or loss) of these additional sequences occurred prior to the separation of Eurotiomycete and Sordariomycete groups. These additional sequences are dispensable for function in *N. crassa* (Jarai and Marzluf, 1990). Homologues of *meaB* are also present in other fungal genomes (Table 1). The predicted proteins encoded by these genes are well conserved, particularly in the DNA binding and dimerization domains (Wong et al., 2008).

Sequence conservation, supported by cross-species complementation studies, suggests that the roles of AreA, NmrA, and MeaB in nitrogen regulation are likely to be largely conserved. However, there are interesting differences even between *A. nidulans* and *N. crassa* that point to some divergence in nitrogen regulation within the filamentous fungi. In *N. crassa*, the levels of *nit-2* mRNA and NIT-2 protein are not regulated (Tao and Marzluf, 1999). In contrast, in *A. nidulans* AreA levels are modulated through autogenous control of *areA* transcription and differential stability of the *areA* transcript (Langdon et al., 1995; Morozov et al., 2000). Deadenylation of the *areA* transcript promotes accelerated turnover of the *areA* transcript under nitrogen-sufficient conditions compared to nitrogen-limiting conditions. This regulated transcript stability is determined by the intracellular levels of glutamine and requires sequences located in the 3′ untranslated region of the *areA* mRNA (Morozov et al, 2000, 2001). Similar 3′ untranslated region sequences have been identified in the *areA* homologues of all *Aspergillus* species, *Penicillium chrysogenum*, *P. roqueforti*, and *P. urticae* but not in *N. crassa* and other Sordariomycetes (Haas et al., 1995; Morozov et al., 2000; Wong et al., 2008). A further difference between *A. nidulans* and *P. chrysogenum* compared to *N. crassa* lies in the proposed function of a second GATA factor that contains an N-terminal GATA domain and a C-terminal leucine zipper domain. These proteins, AreB in *A. nidulans* and NreB in *P. chrysogenum*, are proposed to have a negative role in the regulation of nitrogen-catabolic enzymes (Haas et al., 1997; Conlon et al., 2001). However, a loss-of-function repeat-induced point mutation generated in the *N. crassa* homologue, *asd-4*, prevents sexual development

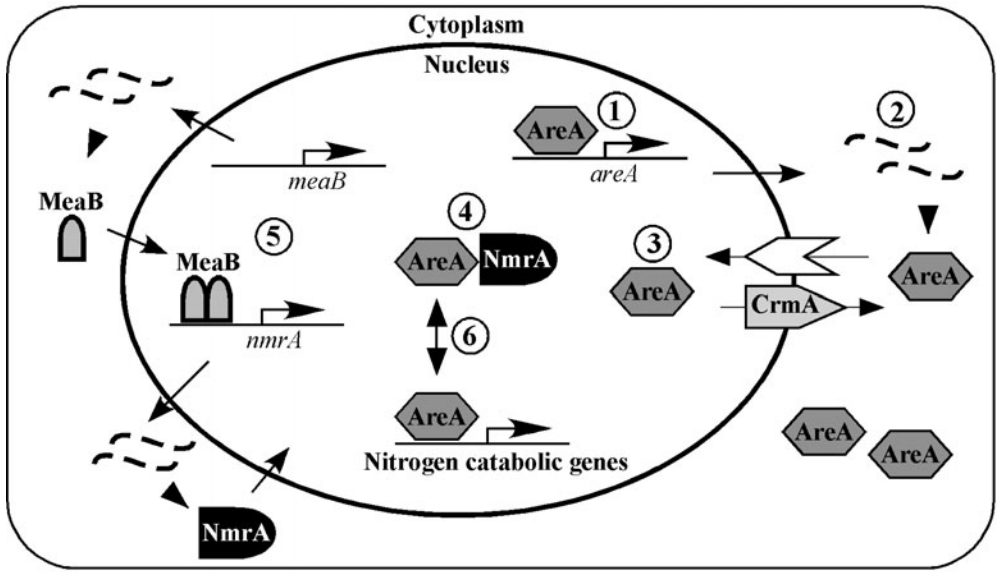


FIGURE 2 Components of nitrogen regulation in *A. nidulans*. The expression of genes subject to nitrogen metabolite repression is regulated by changes in the levels and transcriptional activity of AreA in response to the nitrogen status of the cells (see the text). The levels of *areA* mRNA are influenced by autoregulation of *areA* transcription (1) and differential stability of the *areA* mRNA (2) such that the transcript is degraded more rapidly under nitrogen-sufficient conditions than when nitrogen is limiting or absent. The levels of AreA available to activate transcription within the nucleus are determined by a balance between nuclear import and export (3). Under conditions of nitrogen starvation, AreA accumulates in the nucleus due to a block in CrmA-dependent nuclear export. Once inside the nucleus, the transcriptional activity of AreA is influenced by interaction with the NmrA corepressor (4). The extent to which AreA activity is inhibited by NmrA under nitrogen-sufficient conditions is determined indirectly by the bZIP transcription factor MeaB (5). Under nitrogen-sufficient conditions, MeaB activates *nmrA* expression, leading to increased levels of NmrA and greater inhibition of AreA activity. Thus, the activity of AreA is determined by the relative levels of AreA and NmrA (6), with active AreA predominating under nitrogen-limiting or nitrogen starvation conditions.

without affecting nitrogen-regulated gene expression (Feng et al., 2000). More examples of species-specific variation in aspects of nitrogen regulation in the fungi may emerge as more species are investigated. Already, studies involving the plant pathogen *Fusarium fujikuroi* have revealed some interesting differences with both glutamine and the GS enzyme itself, suggested to play a role in nitrogen regulation (Teichert et al., 2004). In addition, while the *F. fujikuroi nmrA* homologue (*nmr-GF*) can complement both *A. nidulans nmrA* and *N. crassa nmr-1* mutants and *F. fujikuroi* AreA and Nmr have been shown to interact by yeast two-hybrid analysis, deletion of the *nmr-GF* gene does not lead to significant derepression and overexpression does not reduce expression of nitrogen-catabolic enzymes in *F. fujikuroi* (Mihlan et al., 2003; Schönig et al., 2008).

Although *A. nidulans* AreA and *N. crassa* NIT-2 are viewed as global nitrogen regulators, the range of activities under their control may extend beyond nitrogen metabolism. The *F. fujikuroi areA* homologue, in addition to a role in nitrogen control, also regulates certain genes involved in secondary metabolite biosynthesis unrelated to nitrogen metabolism, and *P. chrysogenum* NREA binds to sequences in the promoters of genes involved in penicillin biosynthesis (Tudzynski et al., 1999; Haas and Marzluf, 1995). Screening of *F. verticillioides* microarrays has revealed additional AreA target genes involved in carbohydrate and energy metabolism in *F. fujikuroi* (Schönig et al., 2008). A recent study involving *Magnaporthe grisea* has also highlighted the need for a broader understanding of the integration of nitrogen metabolism with other aspects of cellular metabolism. This study demonstrated that expression of *NMR1*, the *M. grisea* homologue of *nmrA*, is regulated by the trehalose 6-phosphate synthase protein (Tps1) as part of a regulatory complex integrating carbon and nitrogen metabolism through regulation of the pentose phosphate pathway and intracellular NADPH levels (Wilson et al., 2007).

NITRATE ASSIMILATION

The assimilation of nitrate requires the nitrate reductase and nitrite reductase enzyme activities and results in the release of ammonium (Fig. 3). Nitrate is plentiful in soils and can potentially provide a readily accessible source of nitrogen. The acquisition by the filamentous ascomycetes of the nitrate assimilation genes from the oomycetes has been proposed to be an important factor for the success of this group of fungi in soil (Slot and Hibbett, 2007).

The biochemistry of nitrate assimilation is one of the best-studied pathways in fungi and in plants, and the enzymes are highly conserved in structure and function (Crawford and Arst, 1993). Nitrate is actively transported into the cells of *A. nidulans* by two high-affinity nitrate transporters, encoded by *ntrA* and *ntrB*. These genes have redundant functions, and loss of both functions is required

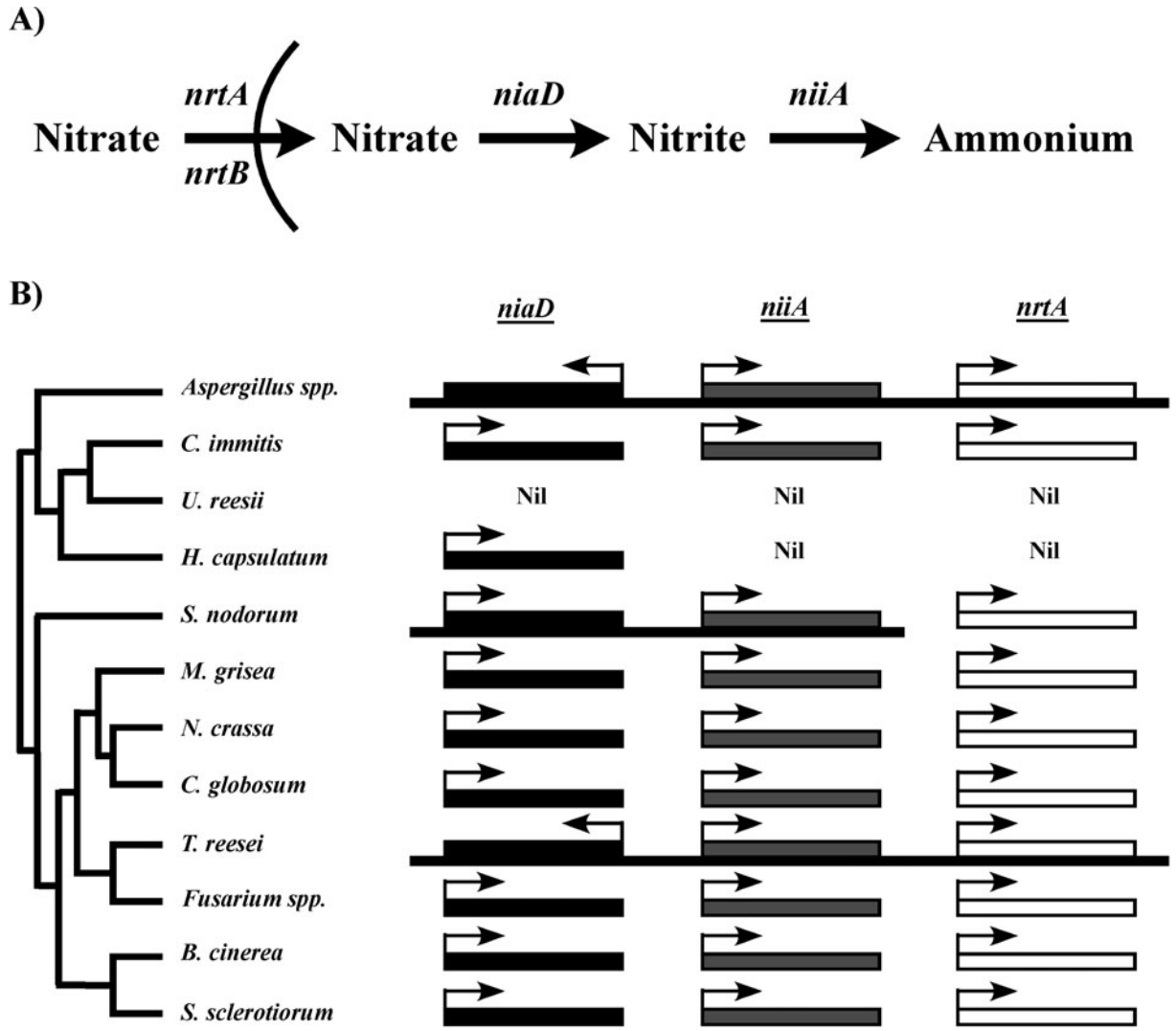


FIGURE 3 Genomic arrangement of nitrate-assimilatory genes in sequenced fungal genomes. (A) In the nitrate-assimilatory pathway, nitrate is transported into the cell by nitrate permeases (encoded by *nrtA* and *nrtB* in *A. nidulans*). Endogenous nitrate is catabolized by nitrate reductase (encoded by *niaD* in *A. nidulans*) to nitrite, which in turn is converted by nitrite reductase (encoded by *niiA* in *A. nidulans*) to ammonium for synthesis of nitrogen-containing biomolecules. (B) Evolution of the nitrate-assimilatory gene cluster in filamentous fungi. The phylogenetic relationships of the various fungi are shown on the left (Fitzpatrick et al., 2006). The *niaD*, *niiA*, and *nrtA* orthologues were identified by blastp and tblastn searches (Altschul et al., 1997) against the respective genome databases, using the *A. nidulans* NiaD, NiiA, and NrtA sequences. The relative orientation of the open reading frames of *niaD* (black rectangles), *niiA* (grey rectangles), and *nrtA* (white rectangles) orthologues in various fungi is indicated by arrows. Thick lines connecting the rectangles indicate that the genes are linked. Nil means that sequence with high similarity could not be found in the respective genome sequences by blastp and tblastn searches. "*Aspergillus spp.*" includes *A. nidulans*, *A. fumigatus*, *A. flavus*, *A. clavatus*, *A. terreus*, *A. oryzae*, *A. niger*, and *Neosartorya fischeri*. "*Fusarium spp.*" includes *F. graminearum*, *F. oxysporum*, and *F. verticillioides*.

to prevent growth on nitrate (Unkles et al., 1991, 2001). Once inside the cell, nitrate is converted to nitrite by a two-step reduction reaction catalyzed by the NADPH-nitrate reductase enzyme. Nitrate reductase structural gene mutants (*niaD* in *A. nidulans* and *nit-3* in *N. crassa*) exhibit specific loss of the ability to use nitrate as a sole nitrogen source. The functional enzyme is a homodimer, with each subunit associated with three cofactors: molybdenum pterin, iron heme, and flavin (Campbell and Kinghorn, 1990). The molybdenum cofactor is shared with the xanthine dehydrogenase enzyme, and its synthesis requires the products of the *cnx* genes (Unkles et al., 1997). The second step in the nitrate assimilatory pathway, the reduction of nitrite to ammonium, is catalyzed by the nitrite reductase enzyme encoded by the *niiA* gene in *A. nidulans* and the *nit-1* gene in *N. crassa* (Marzluf, 1997). The functional importance of the structural domains that coordinate with the cofactors of NiaD and NiiA is indicated by the high levels of

sequence conservation between NiaD and NiiA and their respective orthologues in various other filamentous fungi.

The arrangement of the structural genes in the nitrate assimilation pathway is interesting. In *A. nidulans* the *niaD*, *nii*A, and *nrt*A genes are located together in the genome, and *niaD* and *nii*A are transcribed bidirectionally from a common 1.2-kb intergenic region (Johnstone et al, 1990; Punt et al., 1995). The respective orthologues in various *Aspergillus* species are also present in similar arrangements and orientations. The clustering of these genes may have advantages in coordinating the expression of the two structural genes to efficiently process nitrate into ammonium and to balance the levels of expression of these activities to prevent accumulation of the toxic intermediate nitrite. However, this genomic arrangement is unlikely to be essential for nitrate assimilation, as these genes are found in unlinked loci in many other ascomycetes analyzed (Fig. 3). The presence of the nitrate cluster in the basidiomycete *Ustilago maydis* indicates that this genomic arrangement is the ancestral form and that disassembly of the cluster has occurred in most analyzed ascomycetes. Interestingly, partial rearrangement of the cluster has taken place in *Stagonospora nodorum*, in which the *niaD* and *nii*A orthologues have retained linkage with a different orientation to the ancestral form, while the *nrt*A orthologue is found in a different locus. A similar situation has been described in *Leptosphaeria maculans* (Williams et al., 1995). Furthermore, there appears to be a lack of correlation between the presence and arrangements of the nitrate assimilation genes in various analyzed fungi and their phylogenic relationships (Fig. 3). In the case of *Uncinocarpus reesii*, the nitrate assimilatory genes have been lost entirely, while *Histoplasma capsulatum* has retained only the *niaD* orthologue. In combination, these observations suggest that multiple independent evolutionary events have modified the ancestral nitrate assimilation cluster in the Ascomycota lineage.

The majority of filamentous ascomycetes have lost the clustering arrangement of the nitrate assimilation genes. In the Sordariomycetes lineage, it is puzzling to note that all members have unlinked nitrate assimilation genes, with the exception of *Trichoderma reesei*. Recently Slot and Hibbett (2007) provided evidence that *T. reesei* had regained the complete nitrate gene cluster through horizontal gene transfer from an ancestor of the basidiomycete *U. maydis* after the loss of ability to assimilate nitrate in the Trichoderma lineage. This reacquisition of the cluster was proposed to be advantageous to *T. reesei* in exploiting a new nutritional niche.

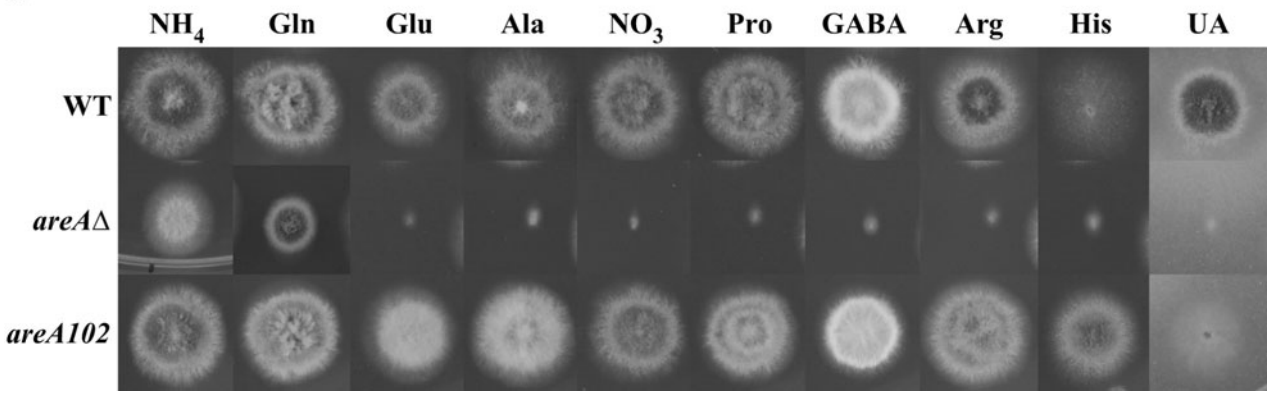
The regulated expression of the nitrate-assimilatory genes is conserved in *A. nidulans* and *N. crassa*, despite their nonconserved genomic arrangement. These genes are expressed only in the absence of ammonium and/or glutamine and in the presence of nitrate. This expression is dependent on the activity of the global nitrogen regulator AreA or Nit2, respectively (associated with the relief of nitrogen metabolite repression), while nitrate induction is mediated by the pathway-specific transcriptional activator, NirA in *A. nidulans* and NIT-4 in *N. crassa*. NirA and NIT-4 contain a conserved N-terminal $Zn(II)_2Cys_6$ binuclear cluster DNA binding domain, bind to similar target sites, and are functionally interchangeable (Yuan et al., 1991; Hawker et al., 1991; Burger et al., 1991a, 1991b; Strauss et al., 1998). Both AreA/NIT-2 and NirA/NIT-4 are necessary for expression of the nitrate-assimilatory genes, and these transcription factors have been shown to directly interact. This association is necessary for DNA binding and for changes in the chromatin state of *niaD-nii*A promoter region that accompanies induction (Feng and Marzluf, 1998; Chiang and Marzluf, 1995; Muro-Pastor et al., 1999; Narendja et al., 2002; Berger et al., 2006). Detailed studies have shown that, in the presence of nitrate, NirA accumulates in the nucleus as a result of nitrate-induced disruption of nuclear export (Bernreiter et al., 2007). However, nuclear retention alone is not sufficient for NirA function and NirA binding requires both nitrate and an active AreA (Berger et al., 2006; Bernreiter et al., 2007). Recent studies have shown that AreA-dependent changes in histone H3 acetylation in response to intracellular glutamine levels, as well as NirA-mediated chromatin remodeling of the *nii*A-*niaD* promoter, are associated with transcription of the nitrate assimilation genes (Berger et al., 2008). Together, induction and nitrogen repression ensure that the nitrate-assimilatory genes are expressed only under conditions in which nitrate breakdown would provide a needed source of nitrogen. A further subtlety in regulation of these genes was revealed by the finding that the stability of the *niaD* and *nii*A transcripts is influenced by nitrate and glutamine availability. Nitrate acts to stabilize these transcripts, reinforcing the transcriptional regulation, and this occurs even in the presence of glutamine, ensuring that nitrate and the toxic intermediate nitrite are removed from the cells (Caddick et al., 2006).

AMINO ACIDS AS NITROGEN SOURCES

The natural habitat of many fungi is the soil, where they play a vital role in the recycling of organic material. Decaying plant and animal material potentially provides a rich source of nutrients, and fungi produce both extracellular and intracellular proteases to break down proteins, releasing amino acids for biosynthesis and as nutrient sources (for examples, see Cohen, 1973; vanKuyk et al., 2000; and Billon-Grand et al., 2002). However, not all amino acids are equally good nitrogen sources and different fungal species exhibit different capacities to extract nitrogen from these substrates (Arst and Cove, 1973; Davis et al., 2005) (Fig. 4). The variation in growth patterns, particularly between closely related species, may reflect differences in the level of synthesis of specific catabolic enzymes and/or permeases. In *A. nidulans* in particular, growth testing on solid media provides a simple and effective qualitative measure of the level of nitrogen-catabolic gene activities. The catabolism of certain amino acids, such as proline and arginine, provides a good source of nitrogen metabolites and supports strong growth in *A. nidulans*, whereas other amino acids, such as histidine and leucine, are very poor sources of nitrogen for the wild-type organism (Fig. 4). Here we consider the pathway of proline catabolism and contrast that with the breakdown of histidine to illustrate different ways in which nitrogen compounds are used.

Proline is broken down to glutamate by two dedicated proline catabolic enzymes encoded by the *prnD* gene (proline oxidase) and the *prn*C gene (L-Δ^1–pyrrolie-5-carboxylate dehydrogenase). The *prn*C and *prnD* genes are contained within a cluster of genes that also encode a specific proline permease (*prnB*) and a transcription factor (*prn*A) that mediates proline induction of the *prn* genes (Jones et al., 1981; Hull et al., 1989). The degradation of proline can provide both a nitrogen source and a carbon and energy source for *A. nidulans*, and the synthesis of these enzymes is regulated by two global regulatory mechanisms: nitrogen metabolite repression mediated by AreA (see "Global Regulation of Nitrogen Catabolism" above) and carbon catabolite repression controlled by the CreA transcriptional repressor (Cubero and Scazzocchio, 1994). The function of CreA is described in detail in chapter 21. A key target of these regulatory proteins is

A



B

Amino acid	Level of growth	
	A. nidulans	*N. crassa*
Glutamine	++++	++++
Ammonium	++++	ND
Glutamate	++	+++
Alanine	++	+
Proline	++	+
Histidine	−/+	−/+
Leucine	+	++
Cysteine	−/+	−/+
Citrulline	+	+++
Lysine	−/+	ND
Isoleucine	+	+
Serine	+++	+
Phenylalanine	+	−/+
Methionine	+	++
Threonine	+	−/+
Valine	+	++
Ornithine	+++	++
Tryptophan	++	−/+
Aspartic acid	++	++++
Tyrosine	++	++
Arginine	+++	+++
Glycine	+++	−/

FIGURE 4 Growth of *A. nidulans* and *N. crassa* on various amino acids as nitrogen source. (A) *A. nidulans* wild-type, *areA*Δ, and *areA102* strains were grown at 37°C for 2 days on ANM solid media (Cove, 1966) containing 1% glucose and ammonium (NH_4), glutamine (Gln), glutamate (Glu), alanine (Ala), nitrate (NO_3), proline (Pro), γ-amino butyric acid (GABA), arginine (Arg), uric acid (UA), or histidine (His) at a final concentration of 10 mM as the sole nitrogen source. (B) Wild-type *A. nidulans* was grown on solid ANM minimal media (Cove, 1966) containing 1% glucose and the indicated amino acid at a concentration of 10 mM. The relative levels of growth after 2 days at 37°C are ranked from strongest (++++) to weakest (−/+). Wild-type *N. crassa* was grown on liquid Vogel's medium lacking NH_4 and NO_3 with 2% sucrose and the indicated amino acid at a concentration of 10 mM. The relative strength of growth is determined by dry mass weight after 3 days of growth at 30°C (Facklam and Marzluf, 1978). The *N. crassa* data are grouped and expressed with symbols as follows: ++++, >30 mg; +++, <30 mg; ++, <15 mg; +, 5 mg; −/+, <2 mg. ND, not determined.

the promoter of the *prnB* gene. Induction is a prerequisite for expression of the *prn* genes, and regulation of the PrnB permease controls the level of proline uptake and, hence, inducer availability (Cubero et al., 2000; Gómez et al., 2003). Proline induction is mediated by the positively acting $Zn(II)_2Cys_6$ transcription factor PrnA (Sharma and Arst, 1985; Cazelle et al., 1998). PrnA contains a tripartite nuclear localization sequence and is constitutively localized in the nucleus (Pokorska et al., 2000; Gómez et al., 2002). Proline is required for the binding of PrnA as a dimer to either

CCGG-N-CCGG or CCGG-6/7N-CCGG repeats and chromatin rearrangements associated with activation of gene expression (Gómez et al., 2002; García et al., 2004). Therefore, the combination of AreA, CreA, and PrnA controls ensures that the proline-catabolic enzymes are strongly expressed under conditions in which proline is available and a source of carbon and/or nitrogen is required by the cells.

In contrast to proline, histidine is an extremely poor nitrogen source and is not a carbon source for wild-type *A. nidulans* (Polkinghorne and Hynes, 1982) (Fig. 4). However, strains carrying the *areA102* mutation can grow strongly on histidine as a sole nitrogen source (Arst and Cove, 1973; Hynes, 1975; Polkinghorne and Hynes, 1982). This difference in phenotype between the wild type and this mutant strain is due to a single amino acid substitution (Leu^{683}-Val) in the GATA DNA binding domain of the AreA transcription factor, which alters the relative affinity of the protein for different promoters and consequently changes expression of its target genes (Kudla et al., 1990; Ravagnani et al., 1997; Starich et al., 1998a, 1998b; Gorfinkiel et al., 1993; Xiao and Marzluf, 1993). The *areA102* mutation is highly pleiotropic, leading to reduced growth on some nitrogen sources and stronger growth on other nitrogen sources, as well as histidine (Arst and Cove, 1973; Hynes, 1975; Diallinas et al., 1995; Davis et al., 1996, 2005). The phenotype of this mutant illustrates how changes in catabolic-gene expression are reflected in the ability of the organism to use particular substrates as nitrogen sources.

The *sarA* gene was identified by mutations that abolished the strong growth of *areA102* strains on histidine and a number of other amino acids but did not reverse the other *areA102* phenotypes (Polkinghorne and Hynes, 1975). This gene was found to encode an L-amino acid oxidase enzyme that is able to release ammonium directly from a wide range of amino acid substrates, producing H_2O_2 and a keto derivative of the amino acid (Davis et al., 2005). Synthesis of the *A. nidulans* L-amino acid oxidase activity requires only AreA function and relief of nitrogen metabolite repression, with no apparent requirement for induction. A similar activity with similar substrate specificity had been identified in *N. crassa*, although the *N. crassa* enzyme encoded by the *lao* gene appeared to require induction (Thayer and Horowitz, 1951; Sikora and Marzluf, 1982; Neidermann and Lerch, 1990, 1991). The level of the L-amino acid oxidase is rate limiting for the catabolism of histidine in *A. nidulans*, as additional copies of the *sarA* gene are sufficient to increase growth of the wild type on this and other amino acids that are substrates (Davis et al., 2005). It is not obvious why higher levels of this activity are not produced by the wild type to maximize the potential yield of nitrogen from histidine and other amino acids. One possibility is that elevated levels of activity and the associated generation of H_2O_2 may impose oxidative stress on the cell. Interestingly, L-amino acid oxidase is a component of snake venom, and H_2O_2 generation contributes to its toxicity (Torii et al., 2000). Under laboratory conditions, increased *sarA* copy number and/or elevated *sarA* expression in *areA102* strains increases growth on these substrates, without any apparent detrimental effects, although even subtle differences may be significant in a natural competitive environment (Davis et al., 2005).

A recent search of the *A. nidulans* genome with the *sarA* sequence revealed the unexpected presence of a closely related paralogue of *sarA* (K. H. Wong, unpublished data). The coding region of the second *A. nidulans* gene is 54% similar to the SarA protein, and the predicted flavin adenine dinucleotide binding domain present in other enzymes of this type is conserved. In comparison, the *N. crassaa* and *Fusarium*

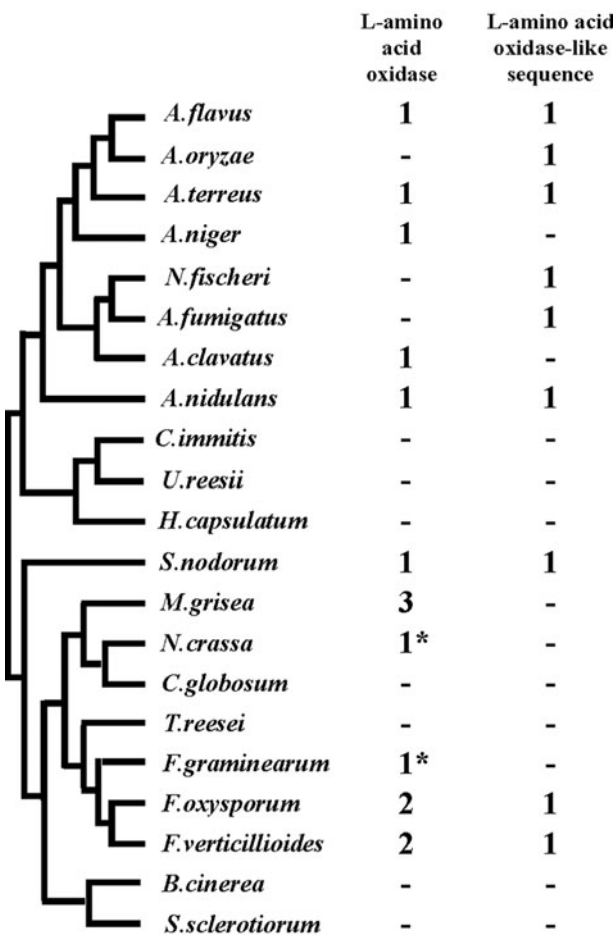


FIGURE 5 Evolution of L-amino acid oxidase in filamentous fungi. The phylogenetic relationships of the various filamentous ascomycetes are indicated on the left (Fitzpatrick et al., 2006; Broad Institute website [http://www.broad.mit.edu/annotation/genome/aspergillus_group/]). L-Amino acid oxidase and related sequences in various fungi were obtained by blastp and tblastn searches (Altschul et al., 1997) of the respective fungal genome database (Broad Institute [http://www.broad.mit.edu/annotation/fgi/]), using the A. nidulans sequences (L-amino acid oxidase, SarA [accession number, AAT84085], and L-amino acid oxidase-like sequence [accession number, EAA64973]). A minus sign (−) represents the absence of any orthologous sequence. An asterisk (*) indicates that the identified sequence was almost equally similar to both L-amino oxidase and L-amino oxidase-like sequences but has a slightly higher percentage of identity and similarity to the indicated sequence. Accession numbers or gene locus (GL) numbers of L-amino acid oxidase sequences are as follows: *A. flavus* (GL, AFL2G_08801.2); *A. terreus* (EAU31805); *A. niger* (CAK45753); *A. clavatus* (EAW12261); *S. nodorum* (EAT79476); *M. grisea* (EDK01261); *N. crassa* (CAD21325); *F. graminearum* (FGSG_13802.3); *F. oxysporum* (GL, FOXG_15820.2 and FOXG_15290.2); and *F. verticillioides* (GL, FVEG_13289.3 and FVEG_12615.3). Accession numbers or GL numbers of L-amino acid oxidase-like sequences are as follows: *A. flavus* (GL, AFL2G_11781.2); *A. oryzae* (BAC55901); *A. terreus* (EAU36894); *N. fischeri* (EAW19737); *A. fumigatus* (EAL86792); *S. nodorum* (EAT82237); *F. oxysporum* (GL, FOXG_05815.2); and *F. verticillioides* (FVEG_03694.3).

graminearum genomes contain a single L-amino acid oxidase orthologue that is intermediate in sequence similarity between the *A. nidulans* SarA and SarA-like sequences (Fig. 5). Searching other fungal genomes for similar sequences, it was found that within the *Aspergillus* genus some species contain only a *sarA* orthologue (e.g., *A. niger*), some possess only the *sarA*-like orthologue (e.g., *A. oryzae*), and some contain orthologues of both *A. nidulans* genes (Fig. 5). Looking beyond this group, some species appear to completely lack either of these genes, whereas others contain multiple copies of the *sarA* orthologue (Fig. 5). The intriguing result from this analysis is the complete absence of any relationship between the presence or absence of *sarA*-related sequences and the phylogeny of these fungi. These genes have been retained, amplified, or entirely lost apparently at random in particular species. It would be very interesting to correlate these differences with the ability of the different fungi to use amino acids such as histidine as nitrogen sources and the various ecological niches that these fungi occupy.

CONCLUDING REMARKS

The free-living lifestyle of many fungi means that the availability and quality of sources of nitrogen are unpredictable and, therefore, the requirement for nitrogen must be met by catabolizing a diverse range of nitrogenous substrates. The synthesis of an equally diverse array of catabolic enzymes is subject to global nitrogen regulation by a class of proteins characterized by a single GATA DNA-binding domain. This motif is present in a number of eukaryotic transcription factors, and indeed there are multiple GATA factors in fungi (Wong et al., 2008). In the filamentous fungi, only one of these factors is responsible for activating catabolic gene expression, and its transcriptional activity is finely modulated in response to the nitrogen requirements of the cells. In the natural world, not only are potential nitrogen sources of variable quality, but also it may not be uncommon for organisms to encounter environments completely devoid of an external source of nitrogen. Studies of *A. nidulans* have shown that complete nitrogen starvation triggers elevated AreA-dependent expression of certain nitrogen-catabolic genes and enhanced nuclear accumulation of AreA (Fraser et al., 2001; Todd et al., 2005). The nitrogen starvation response presumably facilitates the recycling of intracellular nitrogenous compounds and the rapid breakdown of nitrogen sources as they become available. Nitrogen starvation is also likely to be a factor in the initial phases of infection by pathogenic species, where the fungi must penetrate the host tissue before gaining access to intracellular host resources. Furthermore, the growth of the pathogen within the host requires the efficient utilization of these resources and nitrogen limitation can trigger infection-specific gene expression in certain plant pathogenic fungi (for a review, see Snoeijers et al., 2000). The regulatory circuits that control the levels of nitrogen-anabolic or -catabolic enzymes are able to sense and respond to specific signals that reflect the nutritional needs of the cells and the types of nitrogen sources available in a particular environment.

With the availability of multiple fungal genome sequences for analysis, it is becoming clear that there have been extensive changes and rearrangements in the genomes of fungi during their evolution. In some cases it is possible to trace these changes back in evolutionary time by using the phylogeny of the filamentous fungi. In other instances, the connection between evolutionary history and the current state of the genome is less obvious. It is likely that the metabolic versatility of the fungi and the extent to which certain enzymes or pathways have been acquired, conserved, or lost during evolution reflect the diverse environments that this group of organisms have successfully colonized and exploited.

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Amino Acids and Polyamines: Polyfunctional Proteins, Metabolic Cycles, and Compartmentation

ROWLAND H. DAVIS

General metabolism consists of a network of biochemical systems variously linked by enzymes, substrate traffic, and regulatory features. As biochemical genetics took form in the 1940s, beginning with Beadle's and Tatum's methodological breakthrough with the ascomycete *Neurospora* (Beadle and Tatum, 1941), anomalies and complexities began to accumulate that would not fit into the easy comfort of the "one gene, one enzyme" hypothesis. Many of these complexities lay in amino acid and nucleotide pathways. The metabolic pathways themselves are now both well known and widely shared among prokaryotes and eukaryotes. This chapter focuses on three unusual aspects of certain of these pathways: polyfunctional proteins, metabolic cycles, and compartmentation. These features are not unique to fungi, but some of the most detailed analyses originated in fungal systems and have been applied successfully in studies of many other eukaryotes (Davis, 2000).

PATHWAY ANALYSIS

Translation of Eukaryotic mRNAs

At the outset, it should be recognized that a major difference between prokaryotes and eukaryotes lies in the translation of mRNAs. In prokaryotes, multicistronic mRNAs are translated such that each encoded protein is released as it is completed. The stop codon(s) of the proximal sequence is followed by a short untranslated sequence, and ribosomes can initiate translation of the next reading frame at an internal start codon. In eukaryotes, 40S ribosomes bind a 5′ cap of the mRNA and usually initiate at the first AUG codon they encounter as they scan the 5′ upstream sequence. As a general rule, eukaryotic mRNAs have only one coding sequence. However, a number of eukaryotic mRNAs may contain one or more quite short coding sequences between the cap and the main coding sequence. These have evolved to modulate the frequency of initiation of the main coding sequence downstream. In these cases, the small subunit of the ribosome remains attached and scanning, allowing reinitiation at the main coding sequence if proper conditions are met (e.g., whether the 40S ribosome binds the eukaryotic initiation factor 2 [eIF-2] that allows it to recognize the AUG of the main coding sequence). Many of the cases described below are exceptional in that several enzymes might be encoded in an mRNA, but they take the form of multiple domains of a single polypeptide, translated from beginning to end in a single coding sequence.

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Aromatic Amino Acids

The study of aromatic amino acid biosynthesis in *Neurospora crassa* (Fig. 1) provided some of the earliest insights into unusual genetic relationships among related metabolic enzymes. Emerging from this was the appreciation of polypeptide aggregation and gene fusion. When these studies got under way, biochemists became aware of important differences between gene and enzyme organization in eukaryotes and prokaryotes, the latter studies developing at the same time.

The ARO Protein

The *aro* complex of *N. crassa* (Fig. 1) is a set of five genes encoding five enzymes that catalyze the synthesis of chorismic acid from 3-deoxy-D-arabinoheptulosonate phosphate (DAHP) to enolpyruvyl shikimate phosphate (EPSP) (Welch and Gaertner, 1980; reviewed by Davis, 2000). The latter is the precursor of chorismic acid, a common precursor of the aromatic amino acids tryptophan, phenylalanine, and tyrosine. Early mutational studies of *N. crassa* defined four "genes," *aro-1*, *−2*, *−4*, and *−5*, all tightly linked to one another on chromosome II. These were represented by point mutations, which all fell on a linear map of the cluster. Enzymic studies revealed the specific deficiencies in the mutants, but one enzyme of the five required for the overall conversion, dehydroquinase, was not represented by mutation. Among the many phenotypically Aro$^-$ mutants were those with multiple deficiencies, and some of these actually lacked dehydroquinase. Later, a dehydroquinase activity of the quinic acid catabolic pathway (C-dehydroquinase) was shown to be active in many Aro$^-$ mutants and would

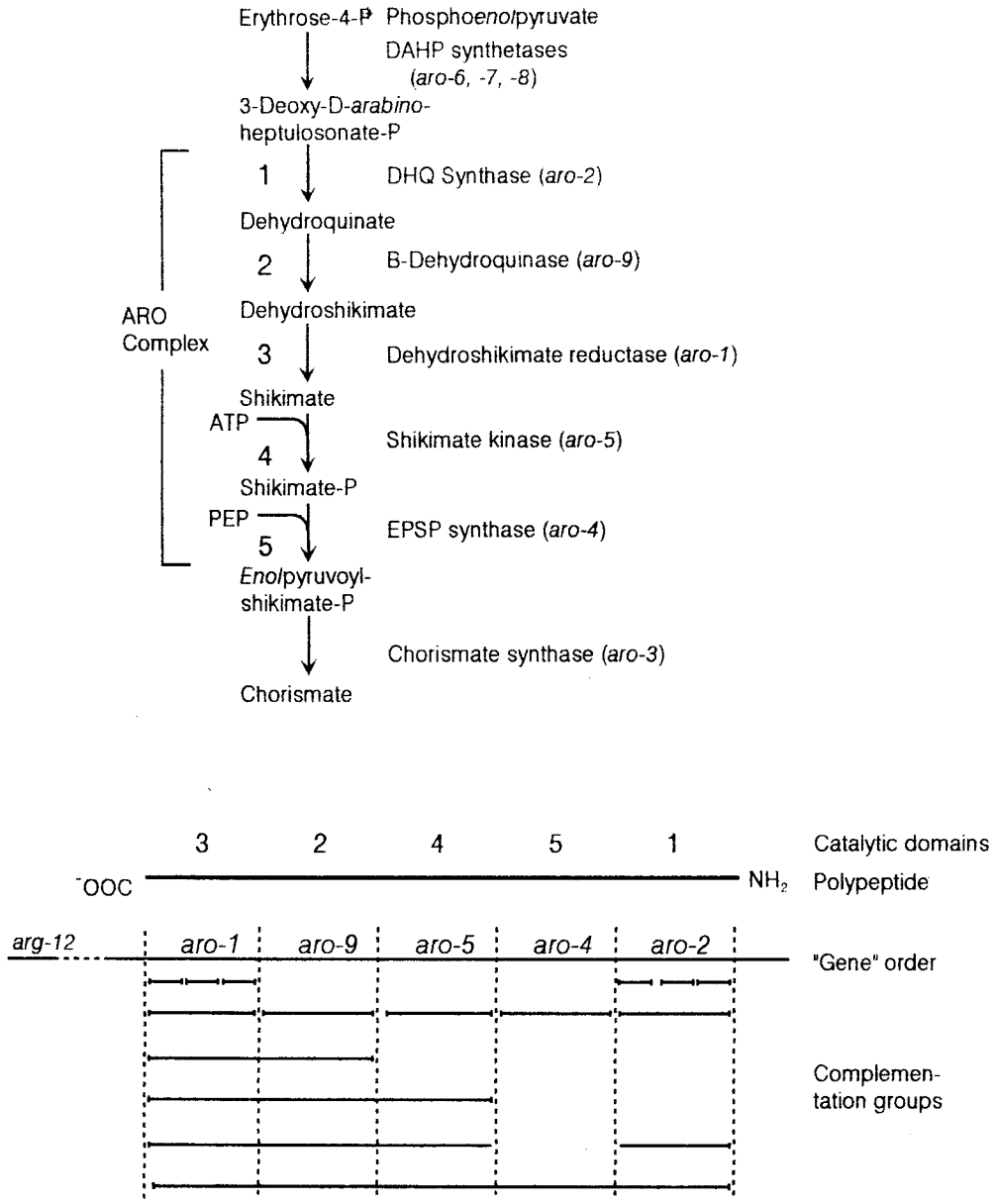

FIGURE 1 Pathway of chorismate synthesis in *N. crassa* (top), showing the metabolic positions of *aro* mutations. The organization of the ARO gene complex on linkage group IIR (bottom) shows the order of the catalytic domains (1 through 5) and of the corresponding component elements of the complex (aro-1, -2, -4, -5, and -9). The complementation groups are represented by lines that group mutants that will not complement; lines that do not overlap signify that complementation will take place between members of the different groups. Note that the translational polarity in this figure is oriented right (N terminal) to left (C terminal). Redrawn from Rines et al., 1969, and reprinted from Davis, 2000.

bypass any deficiency for the biosynthetic enzyme (B-dehydroquinase). Using mutants for the catabolic enzyme, investigators were able to isolate mutations, called *aro-9*, deficient in the B-dehydroquinase. These mutations lay in the *aro* cluster along with the other four *aro* mutations that catalyzed the overall DAHP-EPSP conversion. It was curious, but no more than that, that the map order of the "genes" did not follow the order of the enzymes in the pathway. The *aro* cluster was one of the first genetic systems that was finally recognized as encoding a multidomain protein.

A separate enzyme converting EPSP to chorismic acid is encoded by an unlinked, monofunctional gene, *aro-3*. Finally, the synthesis of DAHP itself, the first substrate dedicated to aromatic amino acid synthesis, is made by any of three isozymes encoded by the genes *aro-6*, *−7*, and *−8*. The three isozymes are individually and allosterically regulated by the cognate end products tryptophan, phenylalanine, and tyrosine, respectively.

Three aspects of these studies, one genetic and two biochemical, have become familiar in other systems involving

multidomain proteins. First, the earliest hypothesis concerning gene-enzyme relations was that the five enzyme activities of the *aro* complex were products of an operon, modeled on the *Escherichia coli lac* operon. At the time, this was reasonable, since few multidomain proteins of eukaryotes had been described in genetic terms. The salient features supporting this model were the tight genetic clustering, the multiple deficiencies of some mutants that suggested multicistronic deletions, and the polarity of some mutations. The last characteristic emerged from a study of complementation among mutants, in which nested overlaps were found: some mutants lacked all five enzymes, some four, some three or fewer, each "deletion mutation" able to complement particular single mutants with its remaining activities. In the *lac* system, mutations with such phenotypes arose either from deletions or from nonsense mutations that blocked progress of ribosomes past the mutant stop codon, with the ribosome failing to reach distal parts of the mRNA before it was degraded. The operon model for the *aro* system was quickly abandoned, as anomalies in the complementation pattern arose, and as analysis of mutant extracts showed that various of the enzyme activities could not be separated by biochemical techniques. The investigators then proposed that the enzymes, as they were being synthesized or after they were synthesized, remained or became obligatorily aggregated, a condition required for the activity of some or all of their activities. Indeed, it was suggested that the complex was a conductor for all of the intermediates of the overall reaction, in which these intermediates did not leave the protein.

The second aspect of the study of the ARO protein complex had to do with more-detailed biochemical studies of the system after 1975. Two groups of investigators applied emerging methods for stabilization of labile proteins (Lumsden and Coggins, 1978; Gaertner and Cole, 1977). Both succeeded in isolating a purified, homogeneous polypeptide, which, as a dimer, catalyzed the entire DAHP-EPSP conversion. The five activities were "domains," as the present language has it, of a single protein encoded by a single, long mRNA. The prior separability of individual enzyme activities and their variable associations with proteins of different molecular weights could be interpreted as interdomain cleavages by proteases or selective inactivation of individual domains. This example of a multidomain protein became a model for others, to be discussed hereafter.

The third aspect of interest in the *aro* gene cluster lay in the catalytic properties of the ARO complex, a property designated "catalytic facilitation" (Welch and Gaertner, 1976). Using the pure ARO protein, the rate of EPSP synthesis in vitro when shikimate, the penultimate substrate, was added directly was compared to the rate of EPSP synthesis when DAHP, the initial substrate, was added. The latter rate was much more rapid, with little lag time of product formation. Two interpretations were possible here. One was that shikimate (and perhaps other intermediates) produced by the complex lay in a compartment shielded from the ambient solution, as previously suggested by Giles' group (see above). The second was that providing the initial substrate of the complex, DAHP, might allosterically change the conformation of later enzyme domains, lowering their K_ms and thus facilitating their catalytic efficiency in nominally low concentrations of intermediate substrates. Some compelling evidence for the latter interpretation was presented, but the two views are not mutually exclusive.

DAHP Synthetases

As noted above, phenylalanine, tyrosine, and tryptophan each feedback inhibit a cognate DAHP synthetase, and each synthetase can individually form DAHP for the common ARO complex. This was one reason mutations for the three synthetases were isolated late in these studies: two synthetases had to be feedback inhibited in order to select auxotrophic mutants lacking the third.

Phenylalanine and Tyrosine

The EPSP produced by the ARO complex is used by the enzyme chorismate synthase to form chorismate, the proximal precursor common to all the aromatic amino acids (Fig. 1). The enzyme, deficient in *aro-3* mutants, is a flavoprotein with an intrinsic flavin reductase. Chorismate is a substrate for chorismate mutase, encoded by the *pt* (phenylalanine + tyrosine) gene to form prephenate (Fig. 2). This is used by

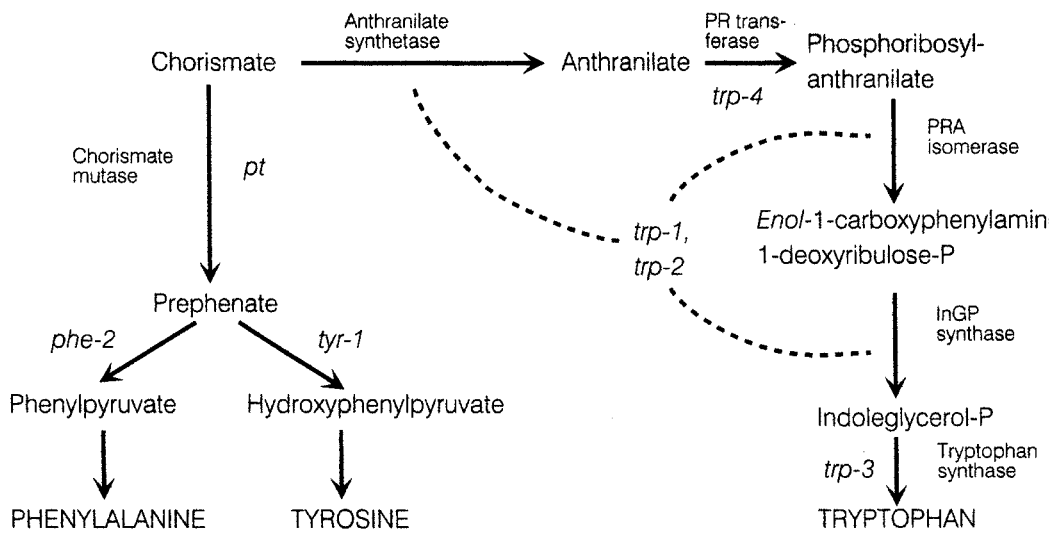

FIGURE 2 Tryptophan, phenylalanine, and tyrosine synthesis in *N. crassa*, showing the metabolic positions of various genes. Note that the complex of *trp-1* and *trp-2* gene products catalyzes three nonsequential reactions. Not all substrates are shown; for details of the tryptophan synthase reaction, see Fig. 3. Reprinted from Davis, 2000.

separate enzymes, one encoded by the *phe-2* gene to form phenylpyruvate (the α-keto precursor of phenylalanine), and the other encoded by the *tyr-1*gene to form hydroxyphenylpyruvate (the α-keto precursor of tyrosine). The two α-keto precursors are converted to the respective amino acids by general transaminases using glutamate.

Tryptophan Synthesis

Tryptophan synthesis depends upon two multidomain proteins, one of them aggregated to a third. The pathway from chorismate to tryptophan has been studied in detail in *N. crassa*, and complementary studies on tryptophan catabolism, which is a part of a potential metabolic cycle, have been performed. Tryptophan in *N. crassa* is a precursor for nicotinic acid and nicotinamide (see Davis, 2000).

Chorismate is used by anthranilate synthase to form anthranilate (Fig. 2), the dedicated substrate for tryptophan. Anthranilate is converted to phosphoribosylanthranilate (PRA) by condensation with 5-phosphoribosyl-1-pyrophosphate (PRPP), a reaction catalyzed by a transferase. PRA is then metabolized to *enol*-1-carboxyphenylamino-1-deoxyribulose-phosphate (CDRP). CDRP is converted to indole-glycerol phosphate and thence to tryptophan. Two polypeptides, specified by the *trp-1* and *trp-2* loci, form an $\alpha_2\beta_2$ heterotetramer required in three reactions in the pathway, the first of which is the anthranilate synthase reaction. The other two activities are the third and fourth reactions, which convert PRA to indole-glycerol phosphate. The last compound is converted to tryptophan via indole, a reaction considered separately below.

The *trp-2* product (the α subunit of anthranilate synthase) can catalyze anthranilate formation alone if ammonia is provided as the N donor. However, the biological reaction uses glutamine as an N donor, and the use of this substrate requires aggregation with the *trp-1* product (the β subunit), which contributes the glutamyltransferase activity via its G domain. In addition, the *trp-1* product has the enzymatic domains for third and fourth reactions; therefore, this subunit is trifunctional if the G domain is taken into account. As in the case of the ARO protein, catalytic facilitation was detected by finding a higher rate of indoleglycerol phosphate formation using PRA rather than CDRP as the initial substrate.

Clearly, gene fusions have taken place in this system if one compares the fungal and bacterial gene organizations. In the latter, most activities are catalyzed by separable proteins. Some fusions may have been selected for catalytic facilitation; others may be fortuitous but not essential (Crawford, 1989). We will revisit this matter in later examples.

Tryptophan Synthase

Tryptophan synthase of *Neurospora* is a multidomain enzyme encoded by the *trp-3* gene. Recognition of the multidomain nature of the enzyme came early, with the observation that mutants carrying some alleles of this gene accumulated indole, while others could use indole as a tryptophan source. Analysis of the enzyme reaction in vitro showed that the overall reaction from indoleglycerol phosphate to tryptophan was indeed the sum of two partial reactions, one forming indole and the other using it. Further analysis demonstrated that the two half-reactions were catalyzed by separate sites on the enzyme (Fig. 3). This was concordant with the more detailed studies of the enzyme of *Salmonella enterica* serovar Typhimurium, in which separate α and β peptides, products

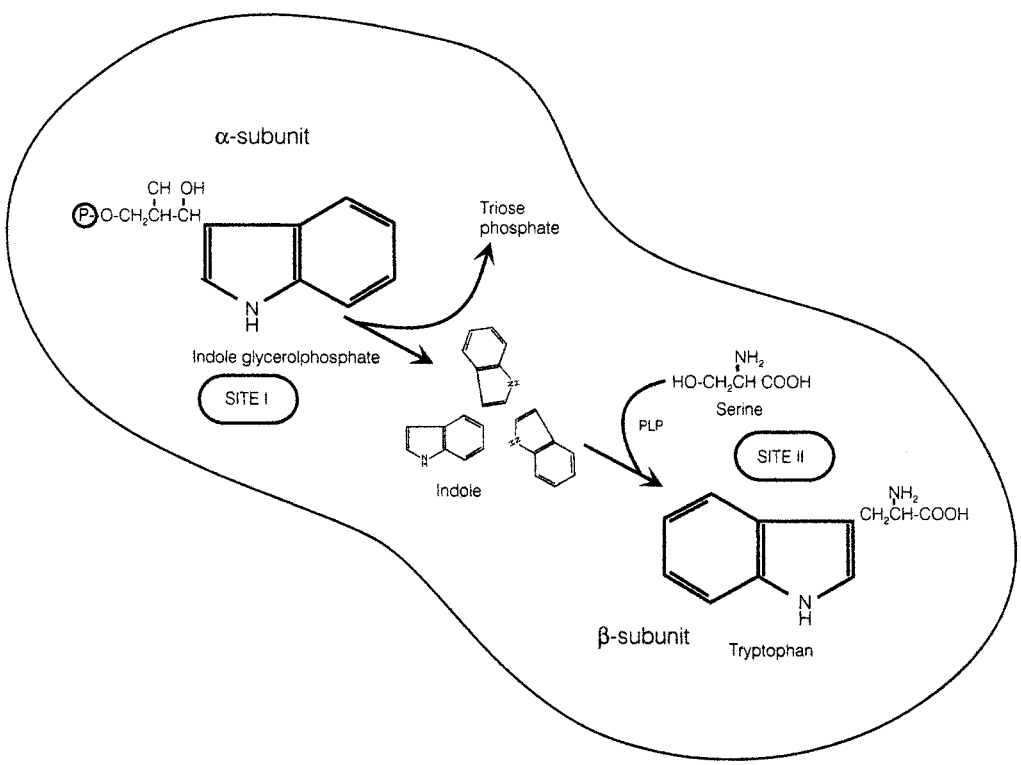

FIGURE 3 The tryptophan synthase partial reactions at sites I (reaction 2) and II (reaction 3). The intermediate indole diffuses between sites I and II, largely confined within the protein. Reprinted from Davis, 2000.

of distinct genes of an operon, carried the corresponding reaction sites (Crawford, 1989). Studies of the reaction in vitro showed that indole appeared not to be a free intermediate of the overall reaction in *N. crassa* but that it was reversibly and noncovalently enzyme bound. Ultimately, structural studies of the *S. enterica* serovar Typhimurium enzyme revealed a tunnel for indole to move from its site of release in the first reaction to the substrate binding site of the second reaction (Burns et al., 1990). An arrangement of fused enzyme domains ensures high local concentration of indole without the need to flood the cell with it. The localization of intermediates in the cell both by multifunctional polypeptides and by enzyme aggregates undoubtedly greatly relieves the solvent capacity of the cell as well as improving the efficiency of enzyme reactions.

A 1:1 stoichiometric ratio of polypeptides that aggregate may be achieved in bacteria by operon organization of the components and in eukaryotes by gene fusion. It is curious that in the case of tryptophan synthase, the order of the two subunits' genes (*trpB* and *trpA* for the β and α subunits, respectively) is reversed in the N-C-terminal order in *N. crassa* (α−β). Artificially fusing the bacterial polypeptides in the α−β order yields an effective enzyme, since the indole tunnel can be achieved in this arrangement if the linking sequence is sufficiently long (Burns et al., 1990).

The Tryptophan Cycle

Tryptophan catabolism, initiated by tryptophan pyrrolase, is notable only for the fact that one of the later products is anthranilic acid, a substrate of the biosynthetic pathway. Copious recycling of anthranilate is minor, however. Some of the immediate products of catabolism, such as indole pyruvate (by deamination), *N*-formylkynurenin, kynurenin, and anthranilate itself, may be excreted, to be used by the biosynthetic pathway only when the catabolic substrate, tryptophan, is exhausted from the medium (Matchett et al., 1968).

Histidine

The histidine pathway consists of 10 steps. In enteric bacteria, all are catalyzed by separate enzymes encoded by an operon. In the fungi *N. crassa*, *Aspergillus nidulans*, and *Saccharomyces cerevisiae*, apparent protein fusion has created a multifunctional gene. In *N. crassa*, this is the *his-3* gene, encoding a polypeptide with three enzyme activities (Minson and Creaser, 1969). The overall pathway starts with phosphoribosylpyrophosphate (required in purine, pyrimidine, and tryptophan biosynthesis), which is converted in the first dedicated step to phosphoribosyl-ATP. The second and third steps, in which phosphoribosyl-AMP and then phosphoribosylformiminoAICAR-phosphate are made, are catalyzed by the *his-3* gene product. These reactions are followed by six further steps, the last of which is histidinol dehydrogenase, the third reaction catalyzed by the *his-3* product. Thus, the activities of this protein are not sequential and can hardly channel intermediates within or on a protein. While this argues against a channeling interpretation, it may allow enzyme-bound phosphoribosyl-AMP to be directed to the next reaction. Moreover, the histidinol dehydrogenase step may be more efficient if the enzyme is part of the larger protein than if it is not (Minson and Creaser, 1969), a possible form of catalytic facilitation. The fact that all reactions are catalyzed by separate enzymes in *S. enterica* serovar Typhimurium suggests that the fungal system has evolved by gene fusion.

Isoleucine and Valine

The precursors of the isoleucine and valine pathways are α-ketobutyrate and pyruvate, respectively (Fig. 4). α-Ketobutyrate is derived from threonine in the threonine dehydratase reaction, and pyruvate is generally available in the cell. From this point on, three enzymes, acetohydroxy acid synthase, a reductoisomerase, and a dehydratase, catalyze the formation of the α-keto precursors of isoleucine (α-keto-β-methyl valerate) and valine (α-ketoisovalerate). These α-keto precursors in turn become isoleucine and valine through activities of general transaminase(s), using glutamate as an amino donor. While the use of the same enzymes for two pathways serves efficiency, it complicates control of the synthesis of the individual amino acids. Feedback mechanisms come into play here; isoleucine inhibits threonine deaminase, while valine inhibits the acetohydroxy acid synthase, but not enough to block isoleucine synthesis. Interestingly, the enzymes are mitochondrial, allowing many of these reactions to proceed in a restricted volume.

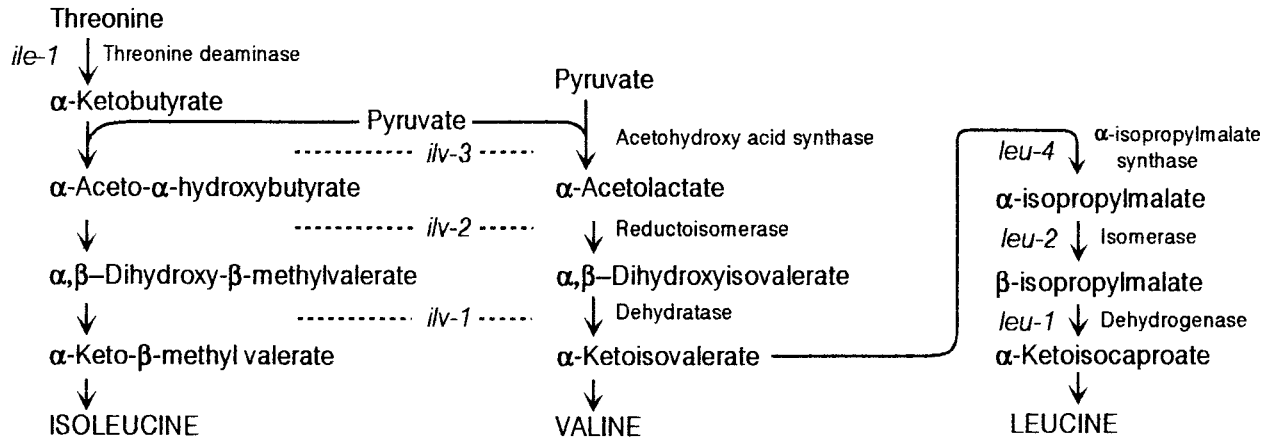

FIGURE 4 Isoleucine, valine, and leucine biosynthesis in *N. crassa*, showing the metabolic positions of mutations. Note that the *ilv-3* (acetohydroxy acid synthase), *ilv-2* (reductoisomerase), and *ilv-1* (dehydratase) genes encode single enzymes that carry out reactions in both the isoleucine and valine pathways. Reprinted from Davis, 2000.

Leucine

A complication also prevails in the aliphatic amino acid pathway: α-ketoisovalerate is the precursor of the leucine pathway (Fig. 4). In excess, valine might therefore partially block leucine as well as valine synthesis. However, the added valine becomes a leucine precursor through the reversible transaminase reaction that provides the required α-ketoisovalerate.

It is of some interest that the leucine pathway, initiated with the synthesis of α-isopropylmalate from α-ketoisovalerate, displays substrate induction of the next two enzymes by positive regulation at the gene level (Gross, 1965). This is true of both yeast and *N. crassa* and requires a specific leucine regulatory protein, encoded by the *leu-3* gene in the latter species. This protein, when bound to α-isopropylmalate, binds the DNA of the enzyme-coding genes and activates transcription. Although other minor allosteric regulatory features have been described in aliphatic amino acid biosynthesis, the ones noted here exemplify the interplay of regulatory mechanisms that must supplement the phenomenon of common enzymes involved in the synthesis of enzymes of three pathways. This theme is played out in other systems. For instance, as noted above, the synthesis of DAHP in the common pathway to tryptophan, tyrosine, and phenylalanine (see above) involves distinct DAHP synthetases, each feedback inhibited by one of the ultimate end products.

METABOLIC COMPARTMENTATION AND CONTROL

As biochemists and geneticists began to appreciate more fully the complexity of metabolism in the 1940s and 1950s, the cellular organization of pathways that might promote efficiency took shape as a new subject. The recognition of feedback inhibition and regulation of gene activity were the first steps in this appreciation. The sense that these mechanisms might generally smooth metabolic traffic within eukaryotic cells derived from studies of bacteria, in which the interplay of these regulatory mechanisms adjusted the synthesis of metabolites to cellular needs and called forth the synthesis of enzymes needed only episodically. Indeed, regulatory mechanisms similar to those in bacteria did prevail in eukaryotes, but several other mechanisms were discovered and pursued in fungal research programs.

Among these was the detection of enzymes with the same function, each dedicated to a specific pathway. The case of three enzymes for DAHP synthesis for aromatic amino acids has been discussed above. A more interesting case involves a second feature of eukaryotic systems, metabolic compartmentation. A well-studied example is that of the two carbamoyl phosphate synthetases, CPS-A and CPSase-P, dedicated to arginine and pyrimidine synthesis, respectively. This case involves both suitable feedback control mechanisms and a means of confining their common product to their respective pathways. Another, described below, is found in the role of mitochondria and vacuoles in managing general arginine and polyamine metabolism.

Carbamoyl Phosphate Synthesis

The simple, unstable intermediate carbamoyl phosphate is required for both arginine and pyrimidine syntheses. In arginine synthesis, it is used in the ornithine carbamoyltransferase (OTCase) reaction to form citrulline. In pyrimidine synthesis, it is a substrate for aspartate carbamoyltransferase (ATCase) and used to form carbamoyl aspartate (Fig. 5). The discovery of an enzyme that catalyzed the formation of carbamoyl phosphate from CO_2, ammonia, and ATP in mammalian liver (Jones et al., 1955) was followed by studies of its role in ammonia detoxification through use by OTCase

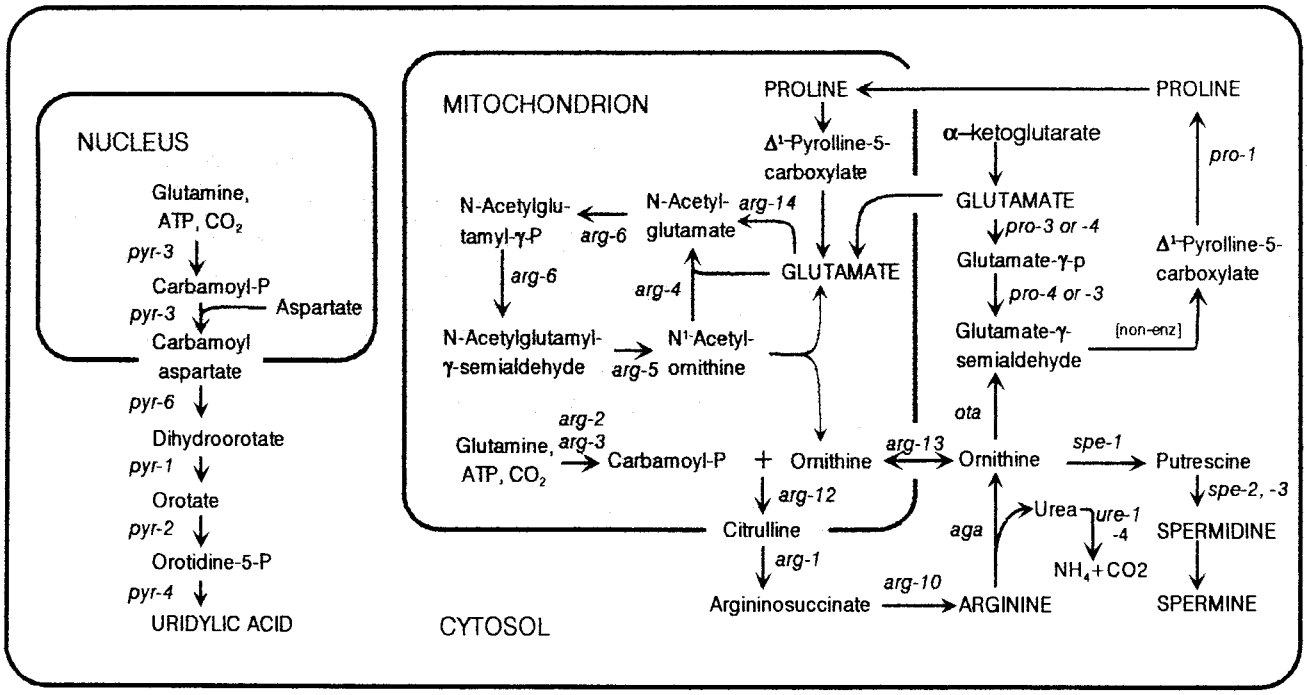

FIGURE 5 The pathways of pyrimidine, arginine, proline, and polyamine synthesis in *N. crassa*, showing the localization of enzymes and the metabolic positions of mutations. See Table 1 for gene-enzyme assignments. Reprinted from Davis, 2000.

and the urea cycle. Until 1962, the actual substrates and indeed several of the biosynthetic CPSases of nonureotelic organisms, including the fungi and bacteria such as *E. coli*, were unknown. While the CPSases then known could use ammonia as an N donor, the in vivo N donor was actually the amido nitrogen of glutamine (Levenberg, 1962). These enzymes were equipped with a small glutaminase subunit or peptide capable of delivering the amido nitrogen of glutamine to the other catalytic sites of the enzyme.

Arginine-Pyrimidine Relationships

Many bacteria have only one biosynthetic CPSase, CPSase I, responsible for the synthesis of this compound. In *E. coli*, pyrimidine nucleotides feedback inhibit CPSase I and ATCase. In order that carbamoyl phosphate remain available for arginine synthesis in conditions of excess pyrimidines, the ornithine accumulated upon feedback inhibition by pyrimidine nucleotides relieves the inhibition of CPSase I sufficiently to satisfy the needs of the arginine pathway. Mutations eliminating the enzyme activity had, as expected, a dual requirement for arginine and pyrimidines. Despite a wealth of mutations in the arginine and pyrimidine pathways isolated in *Neurospora* and yeast in the 1940s and 1950s, none having a dual nutritional requirement was found. Complications attended the mutational analysis of the two pathways, one being suggestive of interactions between arginine and pyrimidine mutations in double mutants. Another was an important difference between yeast and *Neurospora* in the phenotypes of similar CPSase mutants, once they had been recognized as such. The evolution of this problem has been described in detail elsewhere (Davis, 2000).

After considerable difficulty in interpreting the gene interactions noted above, it became clear that two CPSase enzymes existed in *Neurospora*, yeast, and finally, almost all eukaryotic organisms. These enzymes were path specific, at least in terms of their regulatory responses, and were designated CPSase-P and CPSase-A for the pyrimidine and arginine-specific proteins, respectively. Much later, *Bacillus subtilis* was shown to have two path-specific CPSases, each regulated appropriately by the end product of the respective pathway. In this bacterium and in yeast, both CPSases contribute to a common pool, such that a deficiency of one CPS does not block growth; the remaining CPSase can serve both pathways.

The case of *Neurospora* was quite unusual, however. It appeared that the carbamoyl phosphate made by each CPSase was channeled to its own pathway. Mutations of each CPSase imposed a requirement for its own end product. This implied a segregation not only of the two enzymes but also of their products. This channeling, however, was overcome if the use of carbamoyl phosphate in the functional pathway was mutationally blocked by a carbamoyl transferase deficiency (Fig. 6). This was the basis of considerable confusion in the early days but a key to the discovery that two CPSase enzymes existed in the organism.

Carbamoyl Phosphate in Pyrimidine Synthesis

The apparent segregation of the products of each CPSase evoked the question of how a small molecule like carbamoyl phosphate was channeled to a specific pathway. The early genetics of pyrimidine mutants showed that within a single gene, *pyr-3*, one found not only CPSase-P-deficient mutations (later collectively named *pyr-3*M) but also ATCase-deficient mutations (*pyr-3N*). The map positions of the two kinds within the *pyr-3* gene showed some overlap, suggesting that the two sorts of mutations affected a single protein encoding both enzyme activities. The two

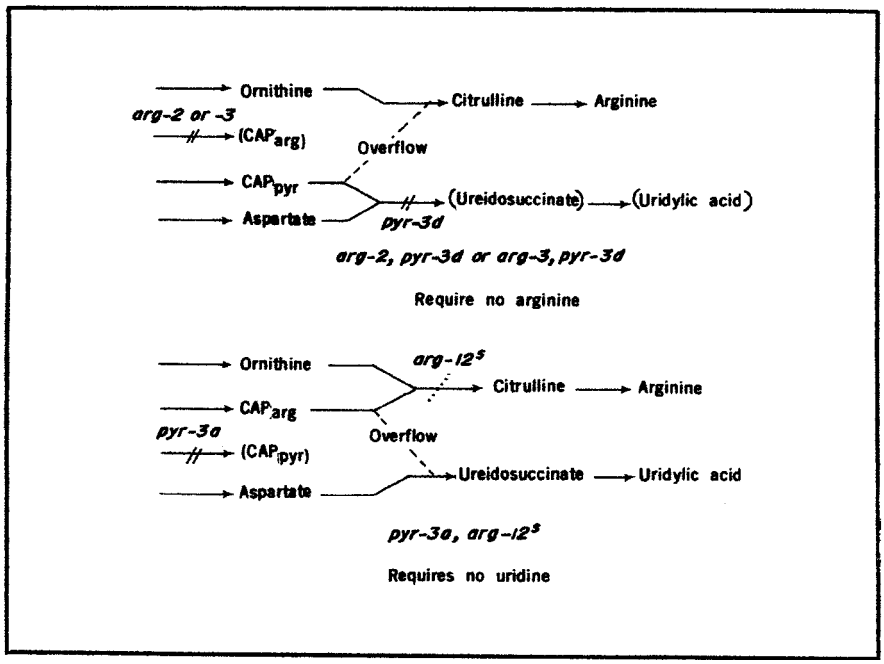

FIGURE 6 Carbamyl phosphate (CAP) overflow between pathways in two types of double mutants of *Neurospora*. Mutations *pyr-3d* (top) and *arg-12*^{s} (bottom) block CAP utilization in one pathway and thereby relieve nutritional requirements imposed by CPS mutants in the other. Parenthesized compounds are not synthesized. CAP is arginine or pyrimidine specific. Reprinted from Davis, 1972.

types complemented one another, but neither complemented with a third type, designated *pyr-3MN*, assumed to lack both functions.

The proposal that a single protein carried both enzyme activities was later proven directly by purification of a bifunctional protein. This was consistent with genetic complementation results, later amplified by showing that *pyr-3M*-type mutations were interspersed with *pyr-3MN*-type mutations at one end of the map; *pyr-3N*-type mutations were found only at the other end. This suggested that the CPSase-P domain was N terminal and the ATCase domain was C terminal (Radford, 1969).

A bifunctional CPSase-P-ATCase protein could in principle (as in the case of tryptophan synthase) retain carbamoyl phosphate between its production by the CPSase-P domain and its use by the ATCase domain. Later work involving yeast provided evidence that this channeling mechanism actually took place in vitro (Lue and Kaplan, 1970), but some doubt has developed about its efficiency. However, a histochemical localization of the protein of *Neurospora*, which liberates the phosphate moiety of carbamoyl phosphate in the second reaction, showed that the reaction product (as a lead precipitate) accumulated in the nucleolus. Later experiments with yeast suggested that this localization might be artifactual and that the bifunctional protein lies in the cytoplasm (Benoist et al., 2000). One therefore cannot with certainty attribute channeling of carbamoyl phosphate of the pyrimidine pathway to its binding the CPSase-P-ATCase protein or to confinement within the nucleus or nucleolus. The path segregation issue was made moot by later analysis of CPSase-A (see below).

Later Steps of Pyrimidine Synthesis

The pyrimidine pathway is common to almost all organisms, including bacteria. The product of ATCase is carbamoyl aspartate, in which a carbamoyl group is transferred to the α-amino group of aspartate. Carbamoyl aspartate is cyclized by the enzyme dihydroorotase to form dihydroorotate; this intermediate is dehydrogenated to form orotate. This is then brought to the nucleotide level by a phosphoribosyl transfer from PRPP. The orotidylic acid formed is decarboxylated to form uridylic acid, which enters general pyrimidine nucleotide metabolism. A few matters of interest here apply to the fungi. First, the dihydroorotate dehydrogenase step is catalyzed by an enzyme on the outside of the mitochondrial inner membrane, a location also found for the mammalian enzyme. Finally, in higher eukaryotes, but not in yeast, the apparent gene fusion of the CPSase-P-ATCase domains implied by the fungal systems in comparison to bacteria is extended in mammals by further fusion of the dihydroorotase. In addition, in mammals, the last two enzymes (phosphoribosyltransferase and decarboxylase) are fused as a bifunctional enzyme.

Arginine-Specific Carbamoyl Phosphate Synthesis

CPSase-A is a heterodimer of two polypeptides encoded by unlinked genes. One, the rather small subunit encoded by the *arg-2* gene, is a glutaminase that yields the amide nitrogen of glutamine as the N donor in the reaction. The other is a larger CPSase polypeptide, encoded by the *arg-3* gene, that uses this moiety, together with ATP and CO_2, to form carbamoyl phosphate. The two polypeptides enter the mitochondrion independently from the cytoplasm and aggregate to form the unstable, mature enzyme. The mitochondrial membrane provides an effective barrier to the exit of normal levels of carbamoyl phosphate to the cytoplasm and nucleus. This was indicated by direct measurement of the arginine-specific carbamoyl phosphate pool remaining in a pyrimidine-starved CPSase-A-deficient *pyr-3M* mutant. In complementary fashion, the mitochondrial membrane blocks entry of small amounts of the pyrimidine-specific carbamoyl phosphate as well, again inferred from the pyrimidine-specific CAP pool remaining in arginine-starved *arg-3* mutants.

The same is not true for yeast. CPSase-P and OTCase are located in the cytoplasm in yeast, thereby obviating channeling of carbamoyl phosphate by the mitochondrial membrane, to be discussed hereafter. As in *B. subtilis*, the carbamoyl phosphate from either pathway of yeast enters a common, cytoplasmic pool, available to both OTCase and ATCase.

Early genetic studies of *Neurospora* turned up a mutation, *arg-12^s*, that overcame the nutritional requirement of a *pyr-3M* (CPS-P-deficient) mutation. The double mutant grew as well as wild-type strains on minimal medium. The *arg-12^s* mutation reduced OTCase activity by over 90%, but the remaining activity supported a normal rate of arginine synthesis. In the double mutant, both ornithine- and arginine-specific carbamoyl phosphates accumulate, and the latter is diverted to ATCase. Similarly, the ATCase deficiency of *pyr3-N* mutants diverts accumulated pyrimidine-specific carbamoyl phosphate to OTCase (Fig. 6). This indicates that at high levels, carbamoyl phosphate of either pathway can penetrate the mitochondrial membrane at a sufficient rate to support the demands of a CPSase-deficient pathway. Each CPSase enzyme is controlled by the end product of its own pathway: UTP feedback inhibits CPSase-P, and arginine severely represses the synthesis of the *arg-2* product. The best indication of the latter is that the double mutant above (*arg-12^s pyr-3M*) acquires a pyrimidine requirement if it is grown in the presence of arginine. These control mechanisms, together with those peculiar to *S. cerevisiae*, are discussed in an integrated treatment of the arginine pathway below.

These studies illustrate, in addition to the possible advantages of enzyme-bound intermediates, another mechanism of metabolic organization, namely, the localization of enzymes and/or substrates within membrane-bound organelles. This contributes to a finer control of metabolite distribution and obviates the need for pancellular concentrations of metabolites sufficient to meet the K_m requirements of individual enzymes.

Ornithine and Arginine Biosynthesis

Arginine synthesis requires many enzymes, organized in two major segments (Fig. 5 and Table 1). A thorough review of arginine metabolism is available (Davis, 2000). The first segment is a cyclic pathway that begins with the synthesis of acetylglutamate from glutamate and acetylcoenzyme A and ends with the deacetylation of acetylornithine to form ornithine. In the latter reaction, the acetyl group is conserved by a transacetylase (ornithine-glutamate acetyltransferase), which, with another molecule of glutamate, regenerates acetylglutamate.

In the second segment of arginine synthesis, ornithine, once released as such, is transformed to citrulline and thence to arginine, most of which is used in protein synthesis in fungal cells grown in minimal medium. A potential problem develops under other conditions. The enzyme arginase catabolizes arginine to ornithine and urea if arginine is provided in excess. Both products of the arginase reaction are further catabolized. The ornithine is transformed

TABLE 1 Gene-enzyme assignments in the arginine, proline, pyrimidine, and polyamine pathways

Gene[a]	Enzyme	Gene ID[b]
aga	Arginase	NCU02333.3
arg-1	Argininosuccinate synthetase	NCU02639.3
arg-2	Carbamoylphosphate synthetase (arginine specific, CPS-A): small subunit	NCU07732.3
arg-3	Carbamoylphosphate synthetase (arginine specific, CPS-A): large subunit	NCU02677.3
arg-4	Acetylornithine-glutamate acetyltransferase	NCU10468.3
arg-5	Acetylornithine transaminase	NCU05410.3
arg-6	Acetylglutamate kinase *and* acetylglutamylphosphate reductase	NCU00567.3
arg-7	Synonymous with *arg-4*	NCU10468.3
arg-8	Synonymous with *pro-3*	NCU01412.3
arg-9	Synonymous with *pro-4*	NCU00106.3
arg-10	Argininosuccinate lyase	NCU08162.3
arg-11	Unknown	Unknown
arg-12	Ornithine carbamoyltransferase	NCU01667.3
arg-13	Mitochondrial arginine-ornithine transporter	NCU02802.3
arg-14	Acetylglutamate synthase	NCU07682.3
ota	Ornithine transaminase	NCU00194.3
pro-1	Δ^1-Pyrolline-5-carboxylate reductase	NCU06471.3
pro-3 or *−4*	Glutamate kinase	NCU01412.3
pro-4 or *−3*	Glutamylphosphate reductase	NCU00106.3
pyr-1	Dihydroorotate dehydrogenase	NCU06532.3
pyr-2	Orotate-PRPP ribosyltransferase	NCU05290.3
pyr-3	Carbamoylphosphate synthetase (pyrimidine specific, CPS-P) *and* aspartate carbamoyltransferase	NCU08287.3
pyr-4	Orotidylate decarboxylase	NCU03488.3
pyr-6	Dihydroorotase	NCU04323.3
spe-1	Ornithine decarboxylase	NCU01271.3
spe-2	*S*-adenosylmethionine decarboxylase	NCU01083.3
spe-3	Spermidine synthase	NCU06727.3

[a]Gene designation in *N. crassa*.

[b]Enzyme name and gene ID designations of the Broad Institute *N. crassa* gene annotation.

to glutamate (via proline), and urea is broken down by urease to carbon dioxide and ammonia. The problem lies in ornithine being a common intermediate of both arginine biosynthesis and degradation, setting up a potential futile cycle. The problem is solved in unusual and different ways in *Neurospora* and yeast, which are now discussed.

The ornithine-arginine pathway attracted the attention of early biochemists for its role in ammonia detoxification in mammals by way of urea synthesis and excretion. Other organisms, including many fungi, have no need for ammonia detoxification and can store and use arginine as a nitrogen source. The first major work on arginine synthesis in the fungi, owing to the large numbers of *Neurospora*, yeast, and *Aspergillus* Arg^- mutants that appeared in early genetic studies, offered a good example of the one gene, one enzyme relationship (Srb and Horowitz, 1944). The quite different patterns of arginine metabolism in fungi and mammals called for a comprehensive study of arginine metabolism in the context of fungal physiology.

Two themes will be followed. The first began with the discovery that all arginine-synthetic enzymes, including OTCase in *N. crassa*, are located in the mitochondrion. This poses the question of how cytosolic arginine might exert, if at all, allosteric control of early enzymes of the pathway, specifically CPSase-A and acetylglutamate synthase and/or acetylglutamyl kinase (Fig. 5). The second theme flows from the discovery that the cells of many fungi (as studied in detail in *Neurospora*) store a great deal of free arginine and ornithine in vacuoles and possess an active arginase. No arginine is catabolized unless it enters cells through the cell membrane or is released from the vacuoles. The role of the vacuole in sequestering arginine and ornithine serves to protect both amino acids from catabolism in the cytosol. In addition, vacuolar sequestration and the properties of the mitochondrial membrane prevent the entry of ornithine arising during arginine catabolism into the biosynthetic pathway in the mitochondrion.

Control of Ornithine Synthesis

The rate-limiting steps in arginine synthesis in minimal medium are the formation of ornithine and carbamoyl phosphate, both of which are controlled in unusual ways. No enzyme of the arginine pathway, except one, the small polypeptide of CPSase-A, is significantly repressed by arginine below levels found in cells grown in minimal medium. Except for CPSase-A, most enzymes are elevated only two- to threefold upon arginine starvation. Allosteric control is exerted only on early enzymes of the acetylglutamate cycle.

Figure 5 shows that cytosolic glutamate enters the mitochondrion, where it may be used by acetylglutamate synthase (the *arg-14* product) and by acetylornithine-glutamate transacetylase (the *arg-5* product). The first of these enzymes, although absolutely essential for arginine synthesis, has low activity and serves to maintain the level of acetylglutamate

during growth and against losses through deacetylation of later intermediates. The transacetylase, on the other hand, maintains the cycle, regenerating acetylglutamate from acetylornithine during each turn.

Acetylglutamate is committed to the synthesis of ornithine by a third enzyme, acetylglutamate kinase, encoded by a distinct domain of the *arg-6* gene (Fig. 5). No arginine is produced endogenously in the presence of excess arginine in the cytosol, owing to inhibition of acetylglutamate synthase and acetylglutamate kinase. The transacetylase is not inhibited by arginine. Investigation of the *arg-14* and the *arg-6* genes revealed unusually complex mechanisms of enzyme synthesis, transport into the mitochondrion, processing of enzyme precursors, and protein interactions enabling feedback control of ornithine synthesis.

As noted above, acetylglutamate kinase is encoded by a multifunctional gene, *arg-6*. This gene also encodes the next enzyme, *N*-acetylglutamate phosphate reductase. These two activities produce acetylglutamyl semialdehyde, the precursor of acetylornithine and ornithine.

The *arg-6* mRNA is translated into a 96-kDa polyprotein, in which the kinase and reductase domains are connected by a 200-amino-acid peptide (Parra-Gessert et al., 1998). The propolypeptide precursor contains an N-terminal mitochondrial targeting sequence and an N-terminal cleavage site just before the N terminus of the mature kinase domain. However, several other internal cleavage sites are found toward the C-terminal end of the connector segment, allowing cleavage of the polyprotein between the two domains as, or just after, it enters the mitochondrion. The mature kinase is an octamer of 51-kDa polypeptides; the reductase is a dimer of two 40-kDa polypeptides. Whether the two enzymes aggregate in the mitochondrion is not known, but curiously, an engineered, uncleaved polyprotein that remains intact in the mitochondrion is catalytically active for the overall reaction.

The acetylglutamate synthase gene, *arg-14*, encodes a 73-kDa protein. It is unusual in having little homology to the bacterial acetylglutamate synthases, unlike the two domains of the *arg-6* protein. It has low abundance in *N. crassa*, in keeping with its quasicatalytic role in maintaining substrate for the acetylglutamate cycle (Yu et al., 1996).

Mutations that eliminate the *arg-6* gene product and certain others that render it inactive also eliminate acetylglutamate synthase activity. This suggests a physical interaction of the *arg-6* and *arg-14* products (Kim and Weiss, 1995). Considerable attention has been devoted to this matter (Glansdorff and Xu, 2007; Abadjieva et al., 2001). One idea is that the kinase and synthase must aggregate to stabilize and impart activity to the synthase. Another is that the kinase and/or reductase proteins have a role in the maturation of the synthase. Finally, because feedback-insensitive mutations of the kinase also affect the sensitivity of the synthase to arginine, arginine can evidently negatively control both enzymes. These matters have been explored in somewhat more detail in the yeast arginine pathway. There, the yeast synthase and kinase do indeed associate physically, and the association is required for synthase activity and stability.

Mutations in the *arg-6* gene rendering acetylglutamate kinase feedback insensitive result in the synthesis of excess ornithine, but this is not accompanied by excess arginine formation (Weiss and Lee, 1980). This reflects a bottleneck arising at the synthesis of carbamoyl phosphate and the diversion of excess ornithine through the catabolic ornithine aminotransferase reaction to proline (Fig. 5).

A remaining question is whether, and if so, how, arginine enters the mitochondrion to exert feedback inhibition. Tests with purified mitochondria reveal that a facilitated diffusion mechanism permits the equilibration of arginine, ornithine, and lysine across the mitochondrial membrane (Yu and Weiss, 1992). This function is probably carried out by the product of the *arg-13* gene in *N. crassa* and the *arg-11* gene of *S. cerevisiae* (Glansdorff and Xu, 2007). Moreover, the three amino acids are mutually competitive and the system has a rather high K_m for each of them. Under circumstances in which cytosolic arginine is low in concentration, little enters the mitochondrion. Under conditions of arginine excess, arginine enters the mitochondrion and can inhibit the early enzyme reactions. Moreover, under these conditions, excess cytosolic arginine blocks the entry of ornithine into the mitochondria and thus its wasteful use in the anabolic pathway. This is one of the few cases in which an organellar membrane has been recruited as a site of feedback inhibition.

In passing, one may note here another element of metabolic organization, namely, derivatization of substrates. Both proline and ornithine syntheses use glutamate as the initial substrate. However, the acetylglutamate cycle effectively isolates glutamate destined for ornithine synthesis via acetylglutamate semialdehyde from the glutamate used for proline synthesis via glutamate semialdehyde.

Control of Arginine-Specific Carbamoyl Phosphate Synthesis

A second question emerged with the discovery of continued carbamoyl phosphate synthesis after addition of arginine to cells grown in minimal medium or to cells previously starved for arginine. Under the latter conditions, CPSase-A is highly derepressed, and carbamoyl phosphate accumulates in mitochondria, where much of it is turned over within the organelle (Davis and Ristow, 1987). Some is diverted to pyrimidine synthesis, a point we can appreciate in phenotypic terms: the double-mutant strain *pyr-3a*, *arg-12*^{s} has no pyrimidine requirement, as noted above. The most striking observation is that if arginine is added to such cells grown in minimal medium, at least two entire doublings take place before a pyrimidine requirement asserts itself, despite the fact that the amount of carbamoyl phosphate accumulated prior to adding arginine can account for little additional growth. Therefore, added arginine does not directly feedback inhibit CPS-A in vivo, consistent with in vitro results. Instead, control by arginine is exerted at the level of enzyme formation. Control is exerted almost wholly on the formation of the small glutaminase subunit of the enzyme, the product of the *arg-2* gene. The larger CPS polypeptide (the *arg-3* product, assayable with ammonia as an N donor) is relatively nonrepressible.

The reduced synthesis of the small polypeptide of CPS-A under conditions of arginine excess is only threefold below those seen in cells grown in minimal medium, but the mechanism of control is unusual. In *N. crassa* and in yeast, the 5′ leader of the mRNA for this protein has a 24-amino-acid upstream open reading frame (uORF). The sequence is translated, and in *N. crassa* excess arginine causes the peptide to bind ribosomes that have translated it. This results in the ribosomes stalling at the uORF termination codon (Wang et al., 1998; Fang et al., 2004), and the same is true for yeast (Gaba et al., 2005). Elimination of the uORF abrogates the negative control of synthesis of the *arg-2* protein by arginine. Arginine-starved cells synthesize the *arg-2* product at a 10-fold-higher rate than

cells grown in minimal medium, not only through relief of the inhibition by the uORF product but also through positive action of the cross-pathway control system on transcription of the *arg-2* gene (see hereafter).

The picture developed here suggests that the continued synthesis of carbamoyl phosphate under conditions of excess arginine might have evolved to the benefit of the organism. With long-term conditions of repression, carbamoyl phosphate accumulates to a point where turnover stabilizes the amount. Carbamoyl phosphate synthesis never stops, owing to the incomplete repression of the small subunit of CPSase-A. Thus, when arginine drops in its concentration and ornithine synthesis immediately resumes, citrulline synthesis will also resume quickly by virtue of residual carbamoyl phosphate and its carbamoyl phosphate synthesis, even as production of the small subunit of CPSase-A resumes at a normal rate.

A quite extraordinary difference exists between the control of arginine synthesis in yeast and the fungal system described above. First, in yeast both CPSase-A and OTCase reside in the cytosol, not in mitochondria. Like the *Neurospora* enzyme, CPSase-A of yeast is not feedback inhibited by arginine, but merely repressed. Second, arginase is induced considerably by arginine addition to yeast cells, unlike the arginase of *Neurospora*, which is constitutive and is induced only threefold at most (Davis, 1986). Third, while ornithine synthesis is controlled similarly to what is observed in *Neurospora*, the ornithine arising through arginase activity would appear in the cytosol, where it might enter the biosynthetic pathway through OTCase. The unusual feature displayed by yeast is the inhibition of OTCase by arginase (Penninckx et al., 1974). The two enzymes, both homotrimers, actually bind in a 1:1 stoichiometric ratio and accomplish a rapid cessation of citrulline synthesis, despite the continued availability of carbamoyl phosphate and ornithine. The cytosolic location of CPSase-A and OTCase and the unusual manner of control of citrulline synthesis are exceptional. *S. cerevisiae* and facultatively anaerobic yeasts display these features, but control in other yeasts and in filamentous fungi resembles that used in *N. crassa*.

The Role of the Vacuole

The understanding of vacuoles of filamentous fungi has peculiar origins. Vacuoles appear in older cells of mycelia in most species and are named as such by resemblance to those of yeast cells and, by extension, to plant cell vacuoles. However, many inclusions of fungal cells had been defined after electron microscopy revealed them, and most were much smaller than what had previously been called the vacuole. Further, even the large yeast vacuole undergoes fragmentation and reconstitutions during cell division in some strains and conditions. The isolation of poorly defined "protease particles" in other studies involving *N. crassa* led to some speculation that they might be vacuoles. The term "vacuole" became somewhat vague as investigators sought adequate and distinctive criteria for the organelle. The most important development in this process came as a surprise from studies of young, rapidly growing germlings of *N. crassa*, in which no vacuoles could be seen by light microscopy or were recognizable in electron micrographs. These studies led finally to isolation from germlings, and purification to homogeneity, of very small vacuoles (Vaughn and Davis, 1981). This allowed development of criteria for their identification, understanding of their osmotic sensitivity, active transport across the vacuolar membrane, discovery of a distinct vacuolar ATPase that regulates its internal pH, an inventory of vacuolar contents, and the physiological role of the vacuole in nitrogen, polyamine, and phosphate metabolism (Davis, 1986). These vacuoles, in their contents and biochemical markers, closely resembled those of yeast except for their size. For an excellent review of the fungal vacuole, see Klionsky et al., 1990.

The interest in the role of the vacuole in arginine metabolism emerged from the observation that arginase, a cytosolic enzyme, produces no urea whatsoever in cells grown in minimal medium despite the existence of a very large cellular arginine pool in even very young cells (nominally about 25 mM in cell water). Indeed, no urea accumulates in cells or the medium of a culture of a ureaseless mutant over many generations. Studies of cellular ornithine, also at high levels in the cells, demonstrated that little of it was catabolized by ornithine aminotransferase, a cytosolic enzyme. During the studies that demonstrated the mitochondrial location of arginine enzymes, sorbitol was used as an osmotic stabilizer of the organelle. Continued studies with extracts of gently disrupted, sorbitol-stabilized cells, followed by differential centrifugation, showed that over 95% of free arginine and ornithine sedimented with a dense, osmotically sensitive particle, distinct from mitochondria. The new particle also contained large amounts of inorganic polyphosphate, the major counterion to the basic amino acids and one of the key attributes of the vacuole of yeast. Indeed, the density of the particles could be attributed to the polyphosphate content and the osmotic loss of vacuolar water during sedimentation in the sucrose density gradient (Weiss, 1973). Plainly, the vacuolar location of arginine and ornithine, which were tightly retained during vacuole isolation, rendered the amino acids unavailable for catabolism (Davis, 1986).

An understanding of the mechanisms that influenced arginine metabolism required knowledge of the quantitative distributions of arginine and ornithine in different cellular compartments. For instance, what are the concentrations of these amino acids in relation to the substrate affinities of the enzymes in the same compartment? Part of the regulatory repertory of the cell consists of the control of the fluxes across the vacuolar and mitochondrial membranes. Analysis of these matters took advantage of radioactive arginine or its intermediates, added to cells at tracer levels. Even at tracer levels, arginine and ornithine are taken up readily through high-affinity uptake systems. The labeled compounds added are so high in specific radioactivity that they do not alter the chemical concentration of the cognate compound in the cells. In exponentially growing cells, chemical determinations can measure the amounts and rates of change of relevant components such as protein and free amino acid pools (including intermediates) independently of tracer addition. Measurement of radioactivity in these compounds, once isolated and assayed, allows one to determine the specific radioactivities (counts per minute per nanomole) of all components. After the addition of radioactive arginine or ornithine, sampling at short intervals allows one to compare the specific radioactivity (counts per minute per micromole) of *new* products of each enzyme reaction with those of the nominal substrates of the enzymes in the cell. Any differences can be attributed to a different distribution in the cell of the metabolically active pool and the remainder of the same compound (Subramanian et al., 1973; Karlin et al., 1976). The arginine, polyamine, and proline pathways are extremely favorable for these studies. The end products are relative dead ends (mostly in the form of protein amino acid for arginine and its intermediates, or

vacuolar pool for arginine and ornithine), and no radioactivity is lost in competing reactions. Unlike many previous experiments in this area, sampling intervals were extremely short (5 to 10 s). This allowed the detection of early and revealing changes in specific radioactivities of each metabolite as the metabolic system proceeded to steady state.

The outcome of these studies is summarized in Fig. 7. In minimal medium, less than 1% of the cellular ornithine lies in the cytosol in exponentially growing mycelia, a steady state maintained by reversible efflux of ornithine from the mitochondria and the uptake of ornithine into, and its release from, the vacuole as the cells grow. In the cytosol, small amounts of ornithine are used for polyamine synthesis (see hereafter) or "leakage" through the catabolic enzyme, ornithine aminotransferase, to the proline pathway. (Catabolism of ornithine proceeds through proline, any excess of which is further catabolized to glutamate.) Similarly, about 1% of the cellular arginine lies in the cytosol, where it is used by arginyl-tRNA synthetase or is taken up by the vacuole. None, as mentioned, is catabolized under these conditions. The concentrations of ornithine and arginine in the mitochondrion and the cytosol are attuned to the measured substrate affinities of the relevant enzymes.

More-refined studies of the fluxes of arginine and ornithine across the mitochondrial and vacuolar membranes in vivo revealed that one-third to one-half of the arginine synthesized by the cell passes through the vacuole on its way to incorporation into proteins (Fig. 7). More dramatic is the determination that one-half of the ornithine made in the mitochondrion and later used by the mitochondrial OTCase actually passes through the cytosol (including entry into, and exit from, the vacuole). This determination was made from specific radioactivity measurements of citrulline synthesized in the mitochondrion: only one-half of the ornithine produced by ornithine-glutamate acetyltransferase is immediately used by OTCase.

The next question is how the vacuolar pools of ornithine and arginine are managed and used during conditions of arginine starvation and excess. Experiments using a prototrophic strain, first grown in the presence of arginine and then transferred to a medium without arginine, yielded insight into the role of the vacuole. Under the initial conditions, an expanded vacuolar pool of arginine forms, ornithine is displaced from the organelle, and considerable hydrolysis of arginine by arginase takes place in the cytosol, providing continued availability of ornithine for catabolism. The amount of ornithine entering the mitochondria becomes severely reduced, owing to inhibition of the entry of ornithine by arginine.

The transition of arginine-replete cells to arginine-free medium is followed by a rapid cessation of arginine and ornithine catabolism and a very slow resumption of ornithine and arginine synthesis. Growth proceeds at the expense of vacuolar arginine, which can continue for at least a doubling of dry weight. In a strain having a feedback-insensitive ornithine pathway, however, ornithine synthesis resumes

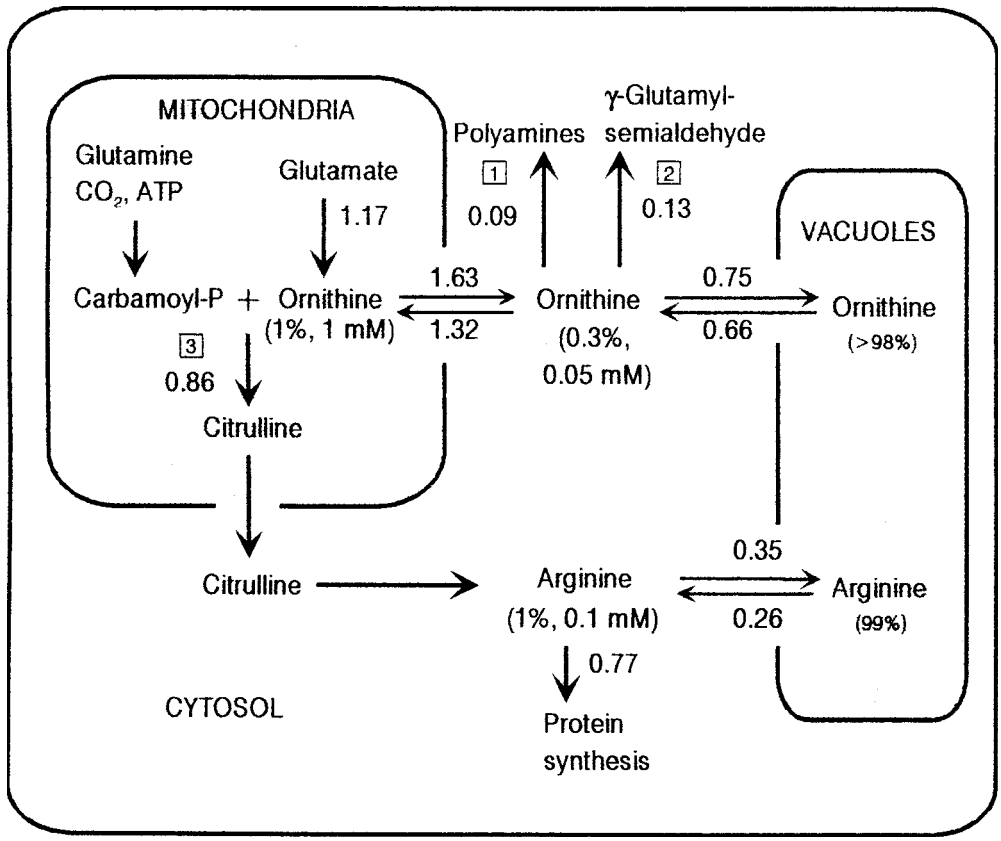

FIGURE 7 Steady-state flux of arginine and ornithine in exponential cultures of *N. crassa* grown in minimal medium. Values are in nanomoles per minute per milligram, dry weight, of mycelium. Boxed numbers identify ornithine decarboxylase (1), ornithine transaminase (2), and ornithine carbamoyltransferase (3). Based on Karlin et al., 1976, and reprinted from Davis, 2000.

rapidly (Goodman and Weiss, 1986). This suggests that in the wild-type strain, feedback inhibition of ornithine synthesis in arginine-replete cells is maintained until the vacuolar pool returns to normal. Indeed, in the transition from arginine-replete to arginine-free conditions, cytosolic arginine concentration quickly diminishes to a level unable to support catabolism of arginine or ornithine but remains high enough to exert continued feedback inhibition. At steady state in arginine-free conditions, feedback inhibition is finally relieved. These and other observations demonstrate that the vacuole plays a major role in adjusting cytosolic arginine concentrations despite large variations in vacuolar arginine content.

The vacuolar transport system has been studied with respect to the carriers and energy requirements (Paek and Weiss, 1989). The proton gradient, maintained by a vacuolar ATPase, supplies the energy for concentrative transport. The arginine carrier has a high K_m for its substrate of about 0.4 mM, in keeping with the range of cytosolic arginine concentrations the vacuole is likely to encounter. It is specific for arginine, inasmuch as ornithine and lysine do not inhibit it.

A broader significance of the vacuolar arginine and ornithine pools lies in the use of these nitrogen-rich amino acids as nitrogen sources. Cells starved for nitrogen become depleted in glutamine, which is thought to be a signal for the release of the vacuolar amino acids (Legerton and Weiss, 1984). Arginine and ornithine are demonstrably released and catabolized in response to N limitation, via proline to glutamate.

Neurospora vacuoles were purified to homogeneity and subsequently made available for many in vitro studies referred to above (Vaughn and Davis, 1981). An inventory of their contents revealed not only arginine and ornithine, but also lysine, histidine, and substantial amounts of long-chain inorganic polyphosphate (Cramer and Davis, 1984). In yeast, the polyphosphate was proposed as an obligatory counterion to the basic amino acids. However, manipulation of phosphate and amino acid levels in *Neurospora* showed this not to be the case, since amino acids could be accumulated in the absence of polyphosphate in vivo. However, the swollen appearance of vacuoles in young, living cells grown under these conditions suggested that polyphosphate was certainly a counterion that greatly reduced the osmotic potential of the small amino acids by a Donnan effect (Van Winkle, 1999). As noted above, vacuoles retain their amino acids during isolation, rendering them the best marker for the intactness of the organelle. At the same time, another compound was detected in the vacuole, namely, spermidine, the major polyamine in most living cells. The significance of this finding is revisited in "Polyamines" below.

CROSS-PATHWAY CONTROL

Most fungi, including yeasts, display a global response to amino acid deprivation. In *N. crassa* and other filamentous fungi, it is referred to as cross-pathway control (Carsiotis and Jones, 1974; Barthelmess, 1982), with many of the genes carrying the *cpc* designation. In *S. cerevisiae*, the system is called general amino acid control, with gene names starting with GC (Hinnebusch, 1988). It is referred to here as cross-pathway control and is described very briefly to fill out the physiological aspects of amino acid synthesis and control. The molecular details of this complex system can be found in a major comparative article on several fungi and references therein (Tian et al., 2007). For a very thorough account of the system in yeast and *N. crassa* up to 1996, see Sachs, 1996.

Starvation of fungi for a single amino acid provokes derepression of enzymes of almost all amino acid biosynthetic pathways as well as certain other pathways. The signal for derepression is a deficiency for the charged aminoacyl-tRNA of the starved pathway. The proximate activator of transcription of the many genes is the product of the *cpc-1* gene of *N. crassa* (CPC1) or that of the *GCN4* gene (Gcn4p) of yeast. These proteins bind to a consensus sequence (5′TGACTCA3′) in the 5′ region of the enzyme-coding genes, and *cpc-1* or *GCN4* mutants fail to derepress under starvation conditions, though they retain basal levels of enzymes. The two genes are regulated mainly through their rates of translation. Translation is modulated by uORFs in the leaders of their mRNAs. When translated efficiently, these uORFs block scanning 40S ribosomes from reaching the main coding region. When inefficiently translated, the termination of translation of the uORFs fails, allowing 40S ribosomes to continue scanning to the main coding region. Removal of the leader renders the *cpc-1* and *GCN4* genes constitutive. To this regulatory system, shared among fungi, are added a number of pathway-specific control genes seen in yeast but not in *N. crassa*. In yeast, these contribute additively to the amplitude of regulation.

Translation of the genes' messages is affected by many other gene products involved directly in protein synthesis, for which mutants have been isolated. Mutants of the *GCN* (general control null) genes of yeast fail to derepress; *GCD* (general control derepressed) mutants cannot be repressed. The major control element is the concentration of eIF-2α, which is needed for 40S ribosomes to recognize AUG start codons and engage the 60S ribosomal subunit. High concentrations of active eIF-2 block ribosome traversal of the uORFs, while a low concentration of eIF-2 renders many 40S ribosomes unable to recognize the last uORF of the set. Amino acid starvation activates a kinase (GCN2p) that phosphorylates and inactivates eIF-2α, thus leading to a reduction in the amount of active eIF-2 in the cell. Much less is known about control of translation of the cross-pathway activator (*cpc-1*) product than is known about Gcn4p.

While the cross-pathway control mechanism is widely shared among fungi and other eukaryotes (Sachs, 1996), the amplitude of regulation varies greatly. In *S. cerevisiae*, the range of regulation is over 10-fold in many cases. In *N. crassa*, individual genes derepress less than fourfold. In both fungi, the amplitude of regulation varies greatly from one enzyme to another; some genes, even in the same pathway, are indifferent to amino acid starvation. The variation is correlated with the number of consensus target sites upstream or within each gene. Yeast has been the most copious source of information of the molecular details of the system, while work in *N. crassa* has mainly provided homologues of several relevant genes, notably *cpc-1* (the *GCN4* homologue) (Paluh et al., 1988) and *cpc-3*, homologous to *GCN2*, encoding eIF-2α kinase (Sattlegger et al., 1998).

The cross-pathway control system points up differences among eukaryotes and prokaryotes in their response to amino acid limitation. In bacteria, the stringent response, involving the production of (p)ppGpp, leads to a blockage of protein synthesis, conserving ATP and slowing growth to permit adaptation to limiting conditions. The quite different response of fungi—elevation of enzymes needed to adapt

to limitation—may reflect the slower growth rate of these organisms.

POLYAMINES

Useful general reviews on polyamines are available (Tabor and Tabor, 1984; Cohen, 1998). Polyamine metabolism in fungi has been studied mainly in *S. cerevisiae* and *N. crassa*. Studies have also been performed with other fungal species, mainly correlating rates of polyamine synthesis with growth, differentiation, and polyamine status, and are reviewed by Hoyt and Davis, 2004.

Functions of Polyamines

The polyamines putrescine, spermidine, and spermine are ubiquitous in eukaryotic cells (and, with the exception of spermine, in prokaryotic cells). As polycations, they bind readily to nucleic acids, polyphosphates, phospholipids, and even to nucleoside triphosphates to the extent that truly free polyamines remain low in cellular concentration. Spermidine is the major polyamine in eukaryotic organisms. Studies of polyamine synthesis have a long history, but the advent of advanced biochemical and molecular techniques has enabled much more refined examination of the synthesis and roles of these compounds. For many years, defining the functions of the polyamines preoccupied mammalian biochemists, and consensus now favors two functions. First, they serve as general cations, comparable to potassium and magnesium, needed for stabilizing nucleic acids to optimize their metabolism and the fidelity of their roles in informational transactions. The cationic charge distribution on these molecules renders them able to fit into nucleic acid helices in ways that inorganic divalent cations do not. Second, spermidine is a participant in a single key biochemical reaction, the formation of hypusine, a compound formed as a covalent modification of a single protein, eIF-5A (Park, 2006) involved not only in protein synthesis but also in several other functions (Feng et al., 2007).

Despite the combination of diffuse roles and one highly specific, essential reaction, polyamines may vary dramatically in amount and rate of synthesis, depending on the stage of growth and environmental circumstances. Ornithine decarboxylase (ODCase) (Fig. 5 and 8), an initial enzyme of the pathway, may vary over a 100-fold range in response to polyamine pool sizes, with transcriptional, translational, and posttranslational mechanisms playing a part. Such an amplitude of regulation suggests either that the enzyme is extremely important in maintaining spermidine levels or that the quantitative excursions of enzyme activity are impossible for the cell to control within more narrow limits. Both views have been favored by particular experimental programs: the first involves mammalian cells, where spermidine synthesis is correlated with growth, particularly in neoplasia (Wallace et al., 2003); the second involves *Neurospora*, in which growth rates are indifferent to severe depletion or excess polyamines (Hoyt and Davis, 2004). In both sorts of organisms, owing to the binding of polyamines to polyanions, little polyamine remains in free solution to exert allosteric or other regulatory influences on ODCase, and the cellular concentration of free polyamines may be extremely labile. In the fungi, the evidence suggests that ODCase is poorly controlled (Davis et al., 1992), given its extravagant variation in specific activity. Evidence for this view is presented below, after a review of the phenomenology of polyamine metabolism and enzyme variation.

Polyamine Synthesis, Transport, and Genetics

Putrescine (1,4-diaminobutryate) is formed by simple decarboxylation of ornithine, a reaction catalyzed by ODCase (Fig. 8). An aminopropyl group is added to one amino group of the diamine to form spermidine. The aminopropyl group is derived from *S*-adenosylmethionine (SAM). SAM is first

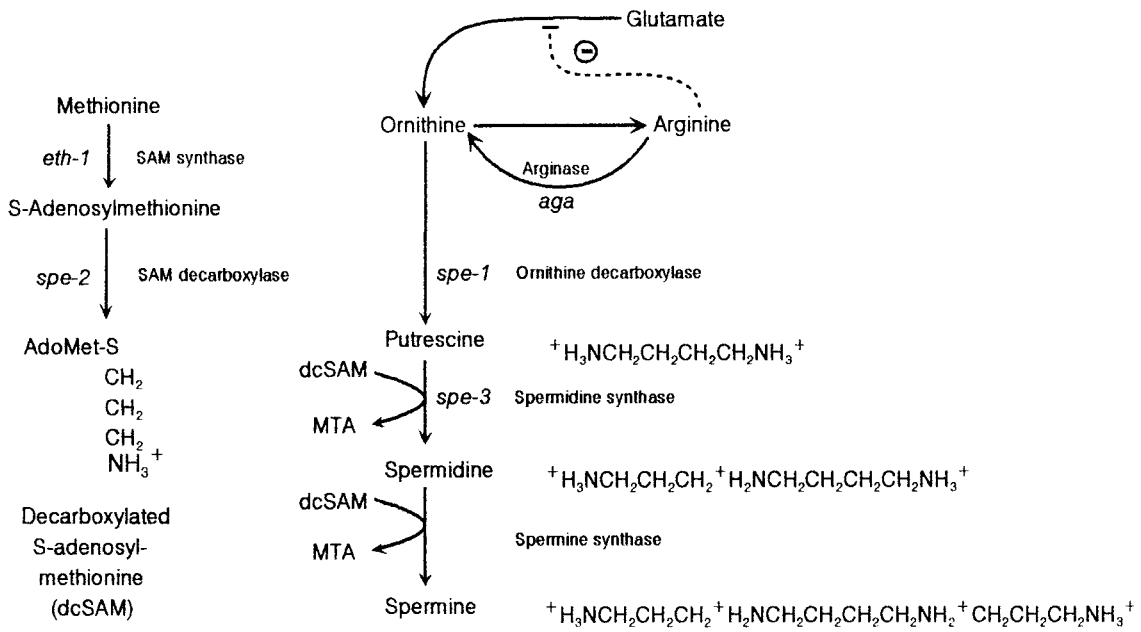

FIGURE 8 Polyamine synthesis in *N. crassa*, with the structures of relevant intermediates and the metabolic positions of mutations. The dotted line signifies allosteric feedback inhibition of arginine synthesis by arginine. MTA, methylthioadenosine. Reprinted from Davis, 2000.

decarboxylated by SAM decarboxylase (SAM-DCase) to form decarboxylated SAM (DC-SAM). An aminopropyltransferase reaction, catalyzed by spermidine synthase, transfers the aminopropyl group of DC-SAM to putrescine. A similar reaction (spermine synthase) converts spermidine to spermine in a second aminopropyltransferase reaction that yields the symmetrical tetra-amine spermine (Fig. 8). In fungi, spermidine is the major polyamine. For example, *Neurospora* contains, per milligram dry weight, 0.8 nmol of putrescine, 18 nmol of spermidine, and 0.4 nmol of spermine. It is of some interest that the SAM-DCase reaction requires putrescine, the other substrate of the first aminopropyltransferase reaction, as a cofactor. In this way, the biosynthetic uses of putrescine and DC-SAM are coordinated.

Very little catabolism of polyamines takes place in *N. crassa*, even at high external amine concentrations. Other fungi catabolize polyamine via polyamine oxidases as a source of nitrogen and a means of recycling polyamines (reviewed by Large, 1992). Polyamine catabolism requires polyamine transport, and curiously, *Neurospora* has a saturable, concentrative transport system for putrescine and spermidine despite the organism's inability to catabolize it. However, the system is inhibited by low amounts of calcium and does not function at all in normal laboratory medium. Even more curiously, the entry of spermidine and putrescine into *Neurospora* cells can take place in laboratory media by a nonsaturable, effectively diffusional means, a feature exploited in studies of intracellular binding of polyamines described hereafter. The nature of this "system," if indeed it is more than leakage through the cell membrane, is poorly defined. Most other organisms display nonsaturable amine uptake, although at lower concentrations of polyamines than is the case in *Neurospora*.

The genetics of the polyamine pathway are straightforward (Pitkin and Davis, 1990). The *spe-1* mutants (ODCase-less) are good auxotrophs, requiring putrescine or spermidine for growth once residual spermidine is depleted to 1/10 of the normal level. *N. crassa spe-2* mutants (deficient in SAM-DCase), when transferred from spermidine-supplemented to minimal medium, grow for a long time at a normal growth rate. In *spe-2* mutants, putrescine accumulates to high levels. As in the *spe-1* mutant, the spermidine pool becomes reduced to about 1/10 of the normal amount, but growth continues normally. The continued growth of the *spe-2* cultures suggests that putrescine can fulfill some of the functions of spermidine up to a point, or at least make the residual spermidine more effective in its role. Obviously the synthesis of hypusine (modifying eIF-5A) would not be possible at very low spermidine concentrations, and it is known that this protein and its modification are essential in yeast. Therefore, *spe-2* mutants finally cease growth in the absence of external polyamines. The behavior of *spe-3* mutants (lacking spermidine synthase) resembles that of *spe-2* mutants. Curiously, all three *spe* loci lie in a 20-centimorgan region of linkage group V.

Finally, mutants (*puu-1*) displaying the saturable transport system by virtue of its insensitivity to calcium are intoxicated by high levels of putrescine, demonstrating an advantage of controlling polyamine transport (Davis et al., 1991). The nature of the *puu-1* gene is not known, but it encodes an essential function: the only known mutant for this gene is a temperature-sensitive lethal.

ODCase Control Phenomenology

The focus of this chapter has been on features of fungi that organize and optimize metabolic systems. The management of polyamine pools has revealed a complex physiological landscape in which enzyme regulation, metabolic flow, small-molecule binding, and vacuolar activity converge. Investigations of polyamine physiology in *Neurospora* have revealed a unique problem of enzyme control by end products largely bound within cells.

The most important technical tool in manipulating polyamine pools in *Neurospora* came to light in studies of *aga* (arginase-deficient) strains. When grown in the presence of arginine, these strains suffer feedback inhibition of ornithine synthesis, combined with an inability to form ornithine from arginine. After addition of arginine to an *aga* culture, the growth rate diminishes to about one-half the normal rate, and the polyamine pools become severely depleted. Continued growth is sustained by the decarboxylation of lysine, an analogue of ornithine, which ODCase uses inefficiently under these conditions. The product of the reaction, cadaverine (1,5-diaminopentane), can be used in place of putrescine to make aminopropyl cadaverine and the corresponding hypusine analogue that modifies eIF-5A. The modified protein is moderately functional.

After the addition of arginine to a culture of the *aga* strain, ornithine is depleted from the vacuole. Before significant changes in the intracellular levels of the putrescine or spermidine pools, ODCase activity and ODCase protein begin to increase abruptly at 50 to 70 times the normal rate, and the specific activity of the enzyme at steady state remains about 70 times that of normal specific activity. After several generations, spermidine and putrescine pools are reduced almost to undetectable levels. Upon adding ornithine to such cultures, the putrescine pool is massively increased, owing to the high ODCase activity. The spermidine pool increases to about 50% above normal, and ODCase activity rapidly decreases with a half-life of 55 min. (The normal generation time of *Neurospora* in these experiments is approximately 150 min at 25°C.) The rapid increase of ODCase activity after arginine addition to the *aga* culture may be through increased synthesis, decreased turnover, or a combination of both. The rapid loss of ODCase activity when ornithine and thus polyamines are restored suggests that turnover may play a part. Another point is that the rapid increase in ODCase synthesis as ornithine is depleted may be a response to a specific and very small, unbound fraction of the pool of one or the other polyamine. If so, one or both pools would be the first to change upon ornithine deprivation.

The first question, regarding the nature of the change of enzyme activity, was answered with immunoprecipitation studies of labeled ODCase protein. They revealed that the half-life of ODCase protein in polyamine-starved cells is approximately 9 h, while the half-life of the protein in cells replete with polyamine is 55 min, approximately a 10-fold difference (Barnett et al., 1988). Moreover, the synthetic rate of ODCase protein is about sixfold greater in polyamine-starved cells than in polyamine-replete cells. Manipulation of the polyamines present in cells undergoing repletion shows that both putrescine and spermidine are effectors of turnover, but the rate of synthesis of new ODCase protein is correlated only with the spermidine pool. Overall, the combination of changes in turnover and changes in the rate of synthesis can explain the 70-fold change in the level of enzyme activity upon polyamine starvation. No allosteric effects or protein modifications need be invoked here; the enzyme activity reflects the amount of immunoprecipitable ODCase protein.

Of some interest is the finding that the turnover of ODCase protein is slower in the presence of cycloheximide

than in its absence. This suggests that the turnover process itself requires protein synthesis. The same finding in other organisms led to the discovery that ODCase degradation is mediated by a short-lived "antizyme," the synthesis of which must be maintained in the ODCase turnover process. No direct evidence for a homologous antizyme protein exists in *Neurospora*, but some evidence based on sequence homology with mammalian antizymes suggests their existence in *Schizosaccharomyces pombe* and certain other yeasts and mycelial fungi (Hoyt and Davis, 2004).

Polyamine Sequestration

The question about polyamine sequestration is more intriguing. The notion that polyamines are not wholly free within the cell is an old one, given their tendency to bind polyanions. For that very reason, extracts of cells cannot be used to verify this hypothesis. In vivo tests of the proposition could be approached in *Neurospora*, because its polyamines are not significantly metabolized beyond the biosynthetic pathway. An isotope experiment, modeled on the arginine/ornithine-labeling study described above, was performed. A tracer level amount of highly labeled ornithine was added to cells, and its metabolism was followed through putrescine and spermidine to spermine. The changes in specific radioactivity of new molecules of the three polyamines were measured in short time intervals. The result was that the new molecules of spermine formed in the interval were much more radioactive than the spermidine extracted from the same cells. The quantitative estimate of the sequestered portion of the spermidine pool was no less than 70% of the total, of which, in follow-up experiments, about one-third was vacuolar. Owing to the exchangeability of spermidine bound to cytosolic anions, the 70% of sequestered spermidine is likely to be a minimal estimate (Paulus et al., 1983).

An inventory of the contents of the vacuole (Cramer and Davis, 1984) revealed not only the basic amino acids, as noted above, but over one-quarter of the cellular spermidine. In cells grown in minimal medium, the spermidine was bound to long-chain inorganic polyphosphate (chain lengths of 200 to 400). In fact, much of the spermidine remained bound to this material in extracts, as shown by gel filtration binding assays. The ionic binding of spermidine and basic amino acids to polyphosphate in the vacuole explains why the intact organelle becomes so dense upon isolation in sucrose gradients, where they lose water as they sediment. In cells, the polymeric nature of the phosphate and its binding of amines reduce the osmotic potential of both molecules.

The foregoing experiments indicate that a minority of the polyamines are free in the cell and that the changes in the concentration of this fraction would be large when cells are starved even briefly for ornithine. A wholly different in vivo test for intracellular spermidine binding was done to confirm this conclusion. It relied on the observation that uptake of polyamines from the normal medium relies wholly on a slow, poorly characterized, but effectively diffusional process. Such a system should allow an equilibration of polyamine concentration inside and outside the cell. A mutant entirely lacking ODCase and thus unable to synthesize spermidine was used for this experiment. The mutant was grown exponentially in different concentrations of spermidine, ranging from 0.5 to 5.0 mM external concentration. The cells were harvested and used to determine the amounts of cellular spermidine, expressed in terms of their concentrations in cell water (2.5 ml/g dry weight). The results (Fig. 9)

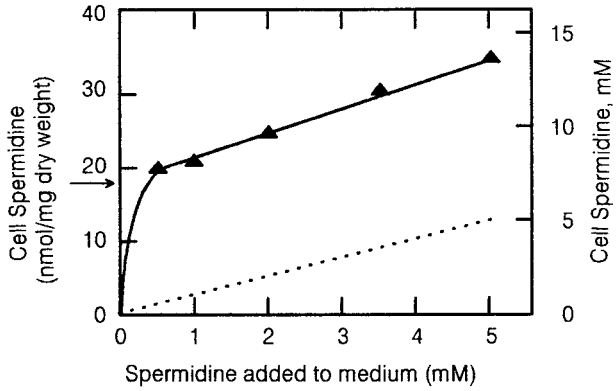

FIGURE 9 Relationship between spermidine concentrations within and outside mycelial cells growing in the presence of different concentrations of spermidine. The strain used carries an ODC-deficient *spe-1* mutation; therefore, all spermidine in the cells originates in the medium. Cellular spermidine is expressed as nanomoles of spermidine per milligram, dry weight (left ordinate) or as millimolar in cell water (2.5 ml per g, dry weight) (right ordinate). The dotted line shows the 1:1 relationship expected of a diffusional equilibrium. Over the range from 0.5 to 5 mM spermidine in the medium, cellular spermidine rises from 9 to ~14 mM (solid line). The arrow on the left ordinate shows the normal concentration of spermidine in wild-type cells grown in minimal medium. Reprinted from Davis, 2000.

reveal that the internal and external concentrations follow a linear relationship between 0.5 and 5.0 mM, as expected for an equilibrating solute. However, the internal polyamine concentration at 0.5 mM external spermidine is nominally almost 8 mM, corresponding to 18 nmol/mg dry weight. This can be attributed to intracellular binding of spermidine. Only after the titration of cellular polyanions does the spermidine concentration equilibrate across the cell membrane. Of great interest is the fact that the amount of intracellular spermidine found when titration is complete is exactly equal to that found in wild-type cells grown in minimal medium (18 nmol/mg dry weight).

One would expect that at least some cellular spermidine would be exchangeable with external spermidine if simple ionic binding to nonvacuolar anions, such as phospholipids, nucleic acids, and polyphosphate, retained spermidine inside the cell. However, although some exchange takes place, this is not at all fast: exchange, if it takes place, is very slow compared to the exchange that takes place when cells are permeabilized immediately after being preloaded with radioactive polyamine. We can attribute the slow rate of exchange in intact cells at equilibrium largely to the cell and vacuolar membranes. Supporting the interpretation of these experiments is that mature, resting cells "excrete" spermidine over longer times, and cells loaded with substantial putrescine also lose some putrescine to the medium (Davis and Ristow, 1989).

The relation of the changes of ODCase activity in response to polyamine pool variations demonstrates that fine-tuning of ODCase activity is unlikely to be workable if only a tiny, labile fraction of polyamines is available as a metabolite or metabolic signal. Therefore, the control of ODCase activity may take other forms, and indeed, that is the case. Both transcriptional and translational mechanisms are at work, and enzyme turnover has been discussed above.

Molecular Studies of Polyamine Enzymes

ODCase and SAM-DCase of *Neurospora* have unusual features that may prove to be general to fungi when other species are tested.

ODCase

The ODCase structural gene of *N. crassa*, *spe-1*, has been cloned and sequenced. The enzyme was purified to homogeneity, and an antibody was raised to it. The enzyme is a dimer of a 484-amino-acid polypeptide, clearly homologous to those of the mouse and yeast. The genes of all eukaryotic species studied are complex, most having a long 5′ sequence encoding an untranslated 5′ leader of the mRNA, matters that are considered below. Bacterial ODCase and arginine decarboxylase sequences do not resemble eukaryotic ODCase gene sequences, nor are they regulated in the same way.

Unlike all eukaryotic ODCase mRNAs studied so far, the abundance of *spe-1* mRNA in *N. crassa* is regulated over a 10- to 12-fold range, which accounts for the changes in the rate of synthesis of ODCase protein (Hoyt et al., 2000a, 2000b). In other eukaryotes, including yeast, the polyamine-regulated variation of ODCase mRNA is much less, and most changes of the synthetic rate of ODCase protein in response to polyamines can be accounted for by changes in the rate of ODCase mRNA translation or cotranslational ODCase turnover prior to dimerization (Toth and Coffino, 1999). In contrast, *N. crassa* ODCase synthesis is regulated in response to polyamines by a combination of transcriptional and translational control. (Changes in turnover account for further variation in the abundance of ODCase protein, as noted above.) The regulation of *spe-1* transcription lies first of all in two control regions upstream of the coding region. An activation region lies in the untranscribed 5′ region upstream of the start of transcription. The second lies in the region encoding the long (622-nucleotide) 5′ untranslated mRNA leader. This region has no effect upon the stability of the mRNA. (The leader has no uORFs.) Second, the 3′ untranslated end of the gene contains a positively acting sequence that is muted in the presence of polyamines. The two upstream elements collectively and the downstream element itself each exert 3- to 4-fold regulation, thereby accounting for the full 10-fold regulation of ODCase mRNA abundance. In addition, a modest translational impediment (threefold) lies in the untranslated leader, and the impediment is relieved mildly under conditions of polyamine starvation. The translational effect is not due to variation in the recruitment of ribosomes to the message (Hoyt et al., 2000a, 2000b).

The phenotype of strains carrying altered *spe-1* genes is of interest. By removing the negatively acting sites of the 5′ untranslated leader, putrescine synthesis is rapid and accumulates to high levels. However, spermidine levels remain only moderately elevated, indicating that another step of the pathway limits spermidine synthesis (Pitkin et al., 1994). This step is SAM-DCase, which initiates the other tributary to spermidine synthesis.

SAM-DCase

SAM-DCase of *N. crassa*, like the enzymes of bacteria and eukaryotes, is a curious enzyme from several standpoints (Hoyt et al., 2000a, 2000b). The transcript of the various genes encodes a proenzyme that is cleaved to form an α subunit and a β subunit. The mature enzyme is an αβ dimer. In *N. crassa*, the proenzyme, estimated to have a molecular mass of 54,721 Da from knowledge of the sequence, yields the β (N-terminal) and the longer α (C-terminal) units of the mature enzyme when cleaved. The *N. crassa* proenzyme contains the amino acid sequence YLLSESSMFV, nearly identical to those of yeast and humans, which includes the cleavage site of other organisms (the E-S bond). Cleavage leaves a pyruvoyl group derived from the serine, an essential cofactor for enzyme activity. Two curious features of the *N. crassa* SPE2 sequence have come to light. The first is that there is no evidence that putrescine stimulates the processing step, a feature shared with the potato proenzyme but unlike the situation in various animals. This is true despite the fungal sequence sharing residues required for putrescine stimulation of cleavage in the latter species. The second is that the fungal (*N. crassa*) sequence is over 100 residues longer than that of the SAM-DCase of most other eukaryotes. The two insertions would not appear to complicate the features of the tertiary structure features required for enzymatic activity of the human enzyme if a similar structure prevails for the fungal enzymes. The insertions are so far seen only in filamentous fungi. SAM-DCase is constitutive, and its activity and its transcript are elevated only three- to fourfold upon spermidine starvation (Hoyt et al., 2000a, 2000b).

CONCLUSIONS

This chapter focuses on several themes that fungal biologists pursued after encountering problematic issues in the early days of biochemical genetics in the 1940s. These had to do with gene fusions and gene clusters, metabolic cycles, and the role of organelles in biochemical organization. None of these themes were unfamiliar to biochemists, but the methodology of genetic dissection of complex problems illuminated all of them in ways that made the fungi attractive as experimental models.

Gene Fusions and Gene Clusters

Speculations about the evolution of multidomain enzymes began in earnest with studies of the tryptophan pathway. There was little doubt that such proteins had formed by fusion of genes, brought about by rare translocations. It was impossible to prove that fusions were necessarily improvements on the original state of separate genes and proteins, particularly if separate polypeptides formed aggregates in the more primitive forms. Such fusions were relatively rare in the amino acid pathways of bacteria, although genes of a given pathway were organized as operons in many cases. At least one advantage of gene fusion would be an assurance of "proper" stoichiometry of the domains involved.

A good deal of argument prevailed about whether intermediates in sequential reactions could be more than fleetingly enzyme-bound in a noncovalent fashion. It was many years before the crystal structure of tryptophan synthase revealed an indole-confining tunnel that facilitated the overall reaction, but few other cases were subjected to rigorous tests. In the meantime, many biochemists pointed to the case of fatty acid synthase of mammals and fungi, which binds intermediates covalently as they are conducted through a series of chain-elongating reactions. This seemed to tell us that this is the way intermediates are effectively bound to multifunctional enzymes catalyzing sequential reactions. The issue of multidomain enzymes as conductors was complicated by the observation that multidomain enzymes often comprise nonsequential reactions as seen in the synthesis of tryptophan or histidine.

The fitness of enzyme colocalization and aggregation in eukaryotic cells can be rationalized in terms of mass action. Even more than in bacteria, the concentration of individual

proteins in eukaryotic cells is quite small, given the larger volume in which proteins lie. By aggregation, enzymes achieve a much higher local concentration with respect to one another, and this alone may facilitate coordinated catalysis. The evidence for protein-protein aggregation accumulated rapidly with the discovery of aggregates that retained their state upon purification and the observation that separate gene products could thereby affect each other kinetically or allosterically. There was no reason to reject the idea that in the cell, many enzymes or polypeptides were aggregated by weak interactions that could not be proved except by cross-linking studies. The lability of the heterodimeric CPS-A of *N. crassa*, a mitochondrial enzyme, hinted at this problem, particularly when compared to the same enzyme of yeast, which is cytosolic and a good deal stabler. The bonds retaining the *N. crassa* polypeptides together need not be as strong as those binding the yeast enzyme owing to the relative volumes in which they exist.

In recent times, the issues of colocalization, protein interaction, and allostery have been seen in evolutionary terms and subjected to more rigorous tests (Kuriyan and Eisenberg, 2007). Colocalization of enzymes may arise through gene fusion, via compartmentation in an organelle, by attachment to the plasma membrane, or by binding cooperatively to a macromolecule such as DNA or RNA. If enzymes are found together, they may interact, and if random translocations bring the corresponding genes together, an in-frame fusion will create a multidomain protein. This, by greatly increasing the local concentrations of the domains, will encourage interdomain interactions and binding, especially if the polypeptide link becomes shorter through later mutational events. This sets the stage for a variety of possible allosteric interactions that increase the fitness or versatility of the protein. Indeed, the evolution of binding interactions between the domains may easily make symmetrical (homo)dimerization possible. The dimeric ARO and CPS-P-ATCase complexes may well have evolved in this way, and the catalytic facilitation proposed for the unit may simply represent conformational changes transmitted through the tertiary structure after initial substrate binding.

The gene fusion event and subsequent evolution of the *arg-6* gene product are not hard to understand, particularly because evidence of a separate N-terminal segment of the second gene persists in the internal linker. The cocompartmentalization of this enzyme with the *arg-14* product is also of interest. The coordinated feedback response of both enzymes when arginine binds to the *arg-6* product can be rationalized in terms of their interaction in the small mitochondrial volume, particularly if one (the *arg-14* product) is attached to the inner mitochondrial membrane.

A systematic study of gene fusion versus gene fission reveals that, over the three domains of life, fusion is four times more likely than fission (Kummerfeld and Teichmann, 2005). The interesting feature of fission, where it is dependably inferred, is that prior association of domains may facilitate the evolution of advantageous domain interaction that will persist after their genetic separation.

Gene clusters, i.e., separate genes of related function found loosely linked on the chromosome, are not of great interest, especially if they are easily translocated without phenotypic consequences. However, certain arrays may be obligatorily coregulated, let us say by a common *cis*-acting site. An example of such a case is found in the tight *qa* (quinic acid degradation) cluster of *N. crassa* genes. The ca. 17-kb region encodes seven polypeptides, two of which are regulatory genes that are divergently transcribed and controlled by a single activating site. Another advantage of clustering of the other, separately controlled *qa* genes may lie in the accessibility of a single large chromatin domain (Howlett et al., 2007) opened as a whole upon induction by quinic acid or related metabolites. Quinic acid is a common product of plant degradation, and its catabolism requires all or most of the functions found in the gene cluster (Walton, 2000). Whatever its origin, such a genetic unit may depend for selfish proliferation upon the ease of lateral transport to related, wood-rotting species by infective DNAs.

Managing Metabolic Cycles

Catabolic pathways for an end product usually do not include intermediates of the biosynthetic pathway. If they do, potential "futile" cycles may result. Various means of preventing this have evolved. One is to make the catabolic substrate able to block (usually allosterically) the enzyme that uses the intermediate, in addition to an enzyme at the head of the pathway. Here, I briefly reiterate one interesting feature of the arginine-catabolic pathway of filamentous fungi and yeast. The catabolic production of ornithine from arginine in the cytosol requires control of its use in the biosynthetic pathway. This is particularly important in view of a constitutive and feedback-insensitive OTCase and continued production of carbamoyl phosphate. This is achieved by blocking the entry of ornithine by the excess arginine into the mitochondria. The importance of this is highlighted by a wholly different mechanism in yeast, in which OTCase and CPS-Aase are cytosolic. There, where arginase is highly induced by its substrate, arginine, arginase physically binds to OTCase and inhibits it.

The Role of Organelles

The evolution of metabolism in eukaryotic cells has involved considerable allocation of enzyme systems and metabolic pools to organelles. The mitochondrion served as an early model of this fact, and it was no surprise that biochemists began to uncover, in the confinement of tricarboxylic acid intermediates to this compartment, the secrets of chemiosmotic ATP production and vectorial transport. The discovery that certain amino acid biosynthetic pathways, such as early arginine enzymes and the isoleucine-valine enzymes, lay within mitochondria showed that energy production was not the sole activity of mitochondria. These examples, among others, increased our appreciation of how membranes could guide metabolic traffic.

The fungal vacuole is another story, but with familiar overtones (Klionsky et al., 1990). The vacuoles of plant cells were widely appreciated early on as the repository of various ions, waste products, nutrients, hydrolytic enzymes, and secondary products. They play an important role in turgor control and thus the structural integrity of plants themselves. The fungal vacuole, easily seen in older cells of many fungal species and very prominent in the yeast cell, was originally considered to be similar to those of plants, but as in plants, the vacuoles are quite diverse in function from one species to another. A second problem with vacuolar definitions is that other membrane-bound organelles—such as the vesicles of the Spitzenkörper, liposomes, exocytotic vesicles, and the Golgi complex, many visualized for the first time with the electron microscope—appeared and further complicated the definition of the vacuole. At this time, the fungal vacuole has reasonably stable operational criteria, despite the diversity of contents. Among these are its specialized ATPase, homologous to the lysosomal ATPase of animal cells, various proteases, α-mannosidase

in the membranes, polyphosphate, and usually amino acids. They are no longer considered passive organelles in any organism, and their activities in amino acid, polyamine, and polyphosphate metabolism in fungi, defined in yeast and *Neurospora*, are a solid testament to that point. Indeed, it is likely that in fungi, as in plants, they have important roles in fine-tuning and otherwise managing metabolic and ionic traffic, varying widely with the species and the environment.

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Cellular and Molecular Biology of Filamentous Fungi
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25

Sulfur, Phosphorus, and Iron Metabolism

JOHN V. PAIETTA

Filamentous fungi provide a fascinating model for how organisms adjust to a constantly changing nutrient environment in order to sustain levels of key elements. The availability of essential macroelements (e.g., phosphorus and sulfur) and microelements (e.g., iron) can be an important limiting factor in the growth of many such organisms. Fungi use complex regulatory networks to monitor macro- and microelement cellular status and specifically up- or down-regulate acquisition and other processes to achieve relatively stable levels (i.e., nutrient homeostasis). Studies in these areas, which have led to the development of current model systems, began with the early biochemical genetic studies of the filamentous fungi.

A primary focus of consideration here is on the varied fungal responses to limitation for iron, phosphorus, and sulfur. Common threads include the versatility and resourcefulness of fungi in the acquisition of these nutrients. For example, a spectrum of transporters (low to high affinity) allows for fungal cells to adjust uptake over a wide range of elemental bioavailability. Further, transcriptional up-regulation of high-affinity transporters is a repeated theme that allows for the scavenging under low and growth-limiting nutrient element levels. Storage and macronutrient homeostasis, including regulatory aspects, are also briefly discussed. While *Aspergillus* and *Neurospora* are considered overall in greater depth here, details of the metabolism of iron, phosphorus, and sulfur from other filamentous fungal species are included throughout. In addition, genomic data now allow for assessment of metabolic similarities and dissimilarities among the fungi, particularly in important emerging experimental systems.

SULFUR

Acquisition of Sulfur: Sulfur Sources

Sulfur is an essential low-abundance element found in a variety of key compounds and macromolecules (e.g., proteins, lipids, electron carriers, and intermediary metabolites) that are required for the growth and function of all cells. Fungi have the versatility to accumulate sulfur atoms in a variety of compounds that are metabolically useful. Subsequent transformation of acquired sulfur into a reduced state (sulfide) allows for the production of sulfur-containing amino acids.

A preferred and relatively abundant sulfur source for fungi is inorganic sulfate. Other inorganic sulfur sources such as sulfite, sulfide, and thiosulfate are also commonly utilized (Paszewski et al., 1994; Thomas and Surdin-Kerjan, 1992). Other than in *Saccharomyces cerevisiae*, studies with elemental sulfur have been confined to demonstration of respiratory reduction in *Fusarium oxysporum* (Abe et al., 2007) and *Phomopsis viticola* (Beffa, 1993). In addition, fungi can obtain sulfate from a number of aliphatic and aromatic sulfate esters (e.g., choline-*O*-sulfate), using a variety of sulfatases such as arylsulfatase and choline sulfatase (Marzluf, 1997; Paietta, 2004). As a typical example, Fig. 1 shows the entry points of the major sulfur sources for *Neurospora crassa* into the sulfur assimilation pathway leading to cysteine formation (Borkovich et al., 2004). The methodical characterization of growth on different sulfur sources has not been widely carried out, but the aggregate of studies demonstrates substantial fungal versatility in obtaining sulfur from the environment.

Release of cysteine and methionine from exogenous protein can also serve as a source of sulfur for many fungi. For example, *N. crassa* secretes an extracellular protease into culture medium whose regulation is responsive to sulfur limitation (also nitrogen and carbon limitation) (Hanson and Marzluf, 1975). The uptake of resulting peptides with cysteine or methionine residues (or directly as free amino acids) satisfies the sulfur requirements of *N. crassa*. Similarly, pathogenic dermatophytes, such as *Trichophyton rubrum*, synthesize proteases responsive to sulfur (and other nutrient) starvation (Apodaca and McKerrow, 1989). An interesting point is that dermatophytes and filamentous fungi such as *Aspergillus fumigatus* can excrete sulfite as a reducing agent to aid in the digestion of keratinous tissues.

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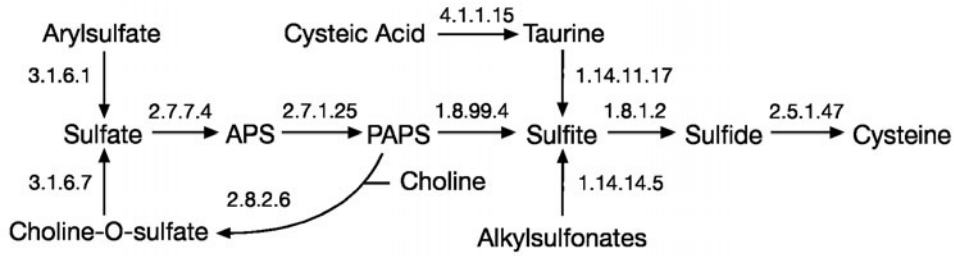


FIGURE 1 Pathway of sulfur acquisition leading to sulfur assimilation and cysteine biosynthesis in *N. crassa*. Potential sulfur sources from the environment or internal stores (e.g., choline-*O*-sulfate) are indicated. EC designations are shown for each pathway step.

The excreted sulfite cleaves the disulfide bonds of keratin and therefore makes the protein accessible for proteolytic degradation (Monod, 2008).

The presence or absence of sulfur compounds in the growth medium is also well known to affect the mycelial-to-yeast-phase transition of certain dimorphic fungi. *Paracoccidioides brasiliensis* (Ferreira et al., 2006) is an example where the presence of cysteine or cystine is required for a transition to the yeast phase, while inorganic sulfate as a sole sulfur source is insufficient.

Besides the sulfur-containing amino acids, other organic sulfur sources such as choline-*O*-sulfate, glucose-6-sulfate, and tyrosine-*O*-sulfate can be transported into the cell and used via appropriate sulfatases (Marzluf, 1997). As an example, arylsulfates would be commonly encountered in soil and organic substrates by fungi. Arylsulfatases catalyze the release of sulfate from a variety of aromatic sulfate compounds depending on the particular specificity of the enzyme. Choline sulfatases can use choline-*O*-sulfate, a storage form of sulfur in fungi, for the production of sulfate (Marzluf, 1997). These sulfatases have a wide, but not universal, fungal phylogenetic distribution and have been shown to be sulfur regulated in a variety of fungi ranging from *N. crassa* (Paietta, 2004) to the plant pathogen *Colletotrichum gloeosporioides* (Goodwin et al., 2000). *N. crassa* has a single arylsulfatase and choline sulfatase, as do a number of other fungal species (Paietta, 2004). For example, *Ustilago maydis* appears to have a single arylsulfatase, which shows low homology to *N. crassa* arylsulfatase but high homology to bacterial arylsulfatases, and a single choline sulfatase (J. V. Paietta, unpublished observations). In contrast, *F. oxysporum* (and related species) has an arylsulfatase that is an ortholog of the *N. crassa* protein but also has at least six sulfatases with homology to the *U. maydis* bacterium-like sulfatase. In total, *F. oxysporum* has 23 proteins classified as sulfatases (Paietta, unpublished). With the caveat that these are homologies and not proven functions, it would be interesting to investigate this battery of potential sulfatases with respect to the pathogenic lifestyle and host range of *F. oxysporum* (and related species). This may reflect a need in the broad plant host range and pathogenic lifestyle to have versatility in accessing sulfur from a variety of available sulfated compounds. The pathogenic "multi-sulfatase" correlation does not appear to extend to mammalian pathogens; for example, *Histoplasma capsulatum* has only a single sulfatase with homology to choline sulfatases (Paietta, unpublished). Fungi lacking arylsulfatase include *S. cerevisiae*, *Schizosaccharomyces pombe* (Borkovich et al., 2004), and the chytrid *Batrachochytrium dendrobatidis*, which does not appear to have any sulfatases with homology to bacterial or other fungal types (Paietta, unpublished).

Acquisition of Sulfur: Transport

The uptake of sulfate into fungal cells is carried out by sulfate permeases, providing a primary sulfur source for the subsequent assimilation pathway (Fig. 1). Most studies on sulfur transport in fungi have focused on sulfate permeases in *S. cerevisiae*, *N. crassa*, and *A. nidulans* (Marzluf, 1997; Pilsyk et al., 2007; Thomas and Surdin-Kerjan, 1997). However, sulfate permeases have a widespread distribution in fungi since homology searches reveal this class of transporter in *F. oxysporum*, *B. dendrobatidis*, *Rhizopus oryzae* (six in total), *U. maydis*, and *Cryptococcus neoformans* (Paietta, unpublished). Additional examples include mycorrhizal fungi, which demonstrate sulfate uptake and increased sulfate supply to the plant (Allen and Shacher-Hill, 2009).

Further, sulfate permease-mediated sulfur acquisition is of importance in industrial antibiotic production. Inorganic sulfate is typically provided as a sole sulfur source to *Penicillium chrysogenum* for industrial penicillin production with involvement of *sutA*- and *sutB*-encoded (primary role) permeases (van de Kamp et al., 2000).

The sulfate permeases (SulP family) usually have a regulatory STAS domain (Rouached et al., 2005) as well as 12 transmembrane spanning domains (Lohi et al., 2002). *N. crassa* has two well-characterized sulfate permeases that are developmentally regulated and subject to derepression upon sulfur limitation. Sulfate permease I (CYS13) is found in conidiospores, while the high-affinity sulfate permease II (CYS14) is expressed in mycelia. Two additional sulfate permeases have been identified in *N. crassa* based on homology (Borkovich et al., 2004) but have not been characterized with regard to sulfate transport. In comparison, *S. cerevisiae* has two sulfate permeases (SUL1 and SUL2) (Thomas and Surdin-Kerjan, 1997) as do *A. nidulans* (sB and astA [Pilsyk et al., 2007]) and *P. chrysogenum* (van de Kamp et al., 2000). Based on homology searches with available genomic data, *R. oryzae* appears to have the largest number of putative sulfate permease genes (RO3G14968, RO3G1128, RO3G14415, RO3G9079, RO3G14248, and RO3G6483 [http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae] (Paietta, unpublished).

Methionine permeases have been less well characterized in fungi. In *S. cerevisiae*, there are at least seven methionine transporters, three of them (AGP3, MUP1, and MUP3) being subject to control by Met4p (Menant et al., 2006). Analysis of the *N. crassa* genome shows three methionine permeases with homology to MUP1 of yeast (Borkovich et al., 2004). However, there has not been further

biochemical characterization since publication of the work establishing a regulated high-affinity methionine transport system in *N. crassa* (Pall, 1971). Homology searches using *N. crassa* high-affinity methionine permease (NCU04942) reveal a wide fungal distribution of putative methionine permeases (e.g., *H. capsulatum* HCAG07082, *Uncinocarpus reesii* UREG02472, *Coprinus cinereus* CC1G08094, *Magnaporthe grisea* MGG03103, and *F. oxysporum* FOXG04496, to include a few [http://www.broad.mit.edu/cgi-bin/annotation/fgi/blast_page.cgi]) (Paietta, unpublished). Both the high-affinity cysteine permease (Yct1) (Kaur and Bachhawat, 2007) and *S*-adenosylmethionine (AdoMet) permease (Sam3) (Rouillon et al., 1999) uptake have been characterized in yeast, and putative homologs exist across the fungal spectrum. Most matches to Sam3 represent general amino acid permeases and so will need biochemical characterization. The bulk of work on cysteine uptake has been carried out with dimorphic fungi, given the importance of cysteine to the mycelial-to-yeast-phase transition (Maresca and Kobayashi, 1989).

Response to Sulfur Limitation and Regulation of Acquisition

Among the filamentous fungi, *N. crassa* and *A. nidulans* have served as primary model systems for sulfur metabolism (Paietta, 2004; Paszewski et al., 2000). The particular focus hereafter is on outlining the representative features of *N. crassa*. As with many other fungi, when *N. crassa* is grown under conditions of sulfur limitation, then an entire set of sulfur-related genes are coordinately derepressed. The sulfur-regulated genes that have been studied most thoroughly encode proteins involved in the transport or hydrolysis of sulfur-containing molecules and include arylsulfatase, choline sulfatase, choline sulfate permease, aromatic sulfate permease, methionine permease, and sulfate permeases I and II (Marzluf, 1997). Typically, derepression of the system occurs by growth on culture medium containing methionine as a sole sulfur source at a low level (0.25 mM). Under high-sulfur conditions (i.e., 5.0 mM methionine) expression of the entire set of sulfur-related genes is repressed. The coordinated expression of such genes is very effective in obtaining sulfur in various forms from the environment. For example, arylsulfatase that is released extracellularly will liberate sulfate from a variety of aromatic sulfate compounds. The resulting sulfate will be transported by coordinately expressed sulfate permease II (mycelial form). Further, aromatic sulfates can be transported into the cell and also be subject to arylsulfatase cleavage.

Basically, the sulfur regulatory system monitors the cellular sulfur status and coordinately up-regulates production of needed proteins as required for sulfur acquisition and provision of an adequate internal supply of sulfur. In contrast, the system is subject to repression under sulfur-sufficient conditions. The *N. crassa* regulatory system can be outlined as follows: (i) the sulfur sensor, which is currently unknown, but may involve the hypothetical *scon-1*$^{+}$ "nuclear limited" gene product (Burton and Metzenberg, 1972) (the nature of the sulfur sensor is still speculative at present, as is the case for many eukaryotic nutrient sensors); (ii) the F-box protein sulfur controller-2 (SCON-2) (also containing a WD40 motif and subject to CYS-3 regulation) and the SCF^{SCON2}-directed control of CYS-3 by targeted degradation (Kumar and Paietta, 1998); (iii) the bZIP transcriptional activator CYS-3, which autoregulates its own expression and binds to the promoter of the *scon-2*$^{+}$ gene and to the entire set of sulfur regulated genes (Paietta, 2004, 2008). A potential end result of the *N. crassa* transduction pathway in signaling the cellular sulfur status would be to change the phosphorylation state of the CYS-3 protein and trigger its targeted destruction as the cells transition to sulfur sufficiency. The established portions of the regulatory system are shown in Fig. 2.

Interestingly, the domain initially termed the "N-terminal domain" in SCON-2 of *N. crassa* (Kumar and Paietta, 1995) was later termed the F-box in yeast and demonstrated to have a role in targeted proteolytic degradation (Bai et al., 1996). Clearly, an important aspect of the sulfur control system in *N. crassa* is the role of the F-box protein SCON-2. Figure 3 shows an alignment of the *N. crassa* SCON-2 protein with a variety of orthologs from diverse fungi displaying the apparent widespread conservation of the system. F-box proteins assemble with Skp-1p, Cdc53p, and Rbx1p to form a complex known as the SCF (Skp1p/Cullin/F-box). SCF complexes act as E3 ubiquitin ligases to target proteins for ubiquitin-mediated proteolysis by the proteasome (Craig and Tyers, 1999). The SCF complex is represented in *N. crassa* by SCON-3 (Sizemore and Paietta, 2002) as the Skp1p homolog, SCON-2 as the F-box protein, NCU06224 as the Rbx1p (ring box) homolog, and an unidentified cullin component. Thus, SCON-2 will bind CYS-3, which would sequester it and block transcription of the sulfur acquisition genes. Subsequently, SCON-2 (with bound CYS-3) interacts with the other needed components to form the SCF^{SCON2} complex, which targets CYS-3 for degradation. A potential useful related therapeutic finding is that the ortholog of SCON-3 in *Microsporum canis*, sconC, is up-regulated by fluconazole exposure and inhibits sulfur metabolism in that dermatophyte (Uthman et al., 2005).

In *A. nidulans*, another well-characterized filamentous fungal system, MetR (Natorff et al., 2003), is the transcriptional activator that corresponds to *N. crassa* CYS-3 and *S. cerevisiae* Met4p (Thomas and Surdin-Kerjan, 1997). MetR and CYS3 exhibit substantial homology only in the DNA binding and dimerization domain (Paietta, 2004). In addition, unlike the highly regulated *cys-3*$^{+}$ gene, expression of *metR* is not affected by sulfur level (Natorff et al., 2003). Other *A. nidulans* components include SconB (Natorff

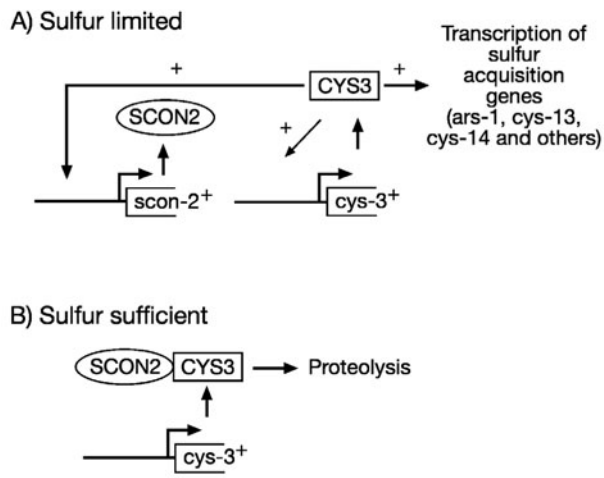
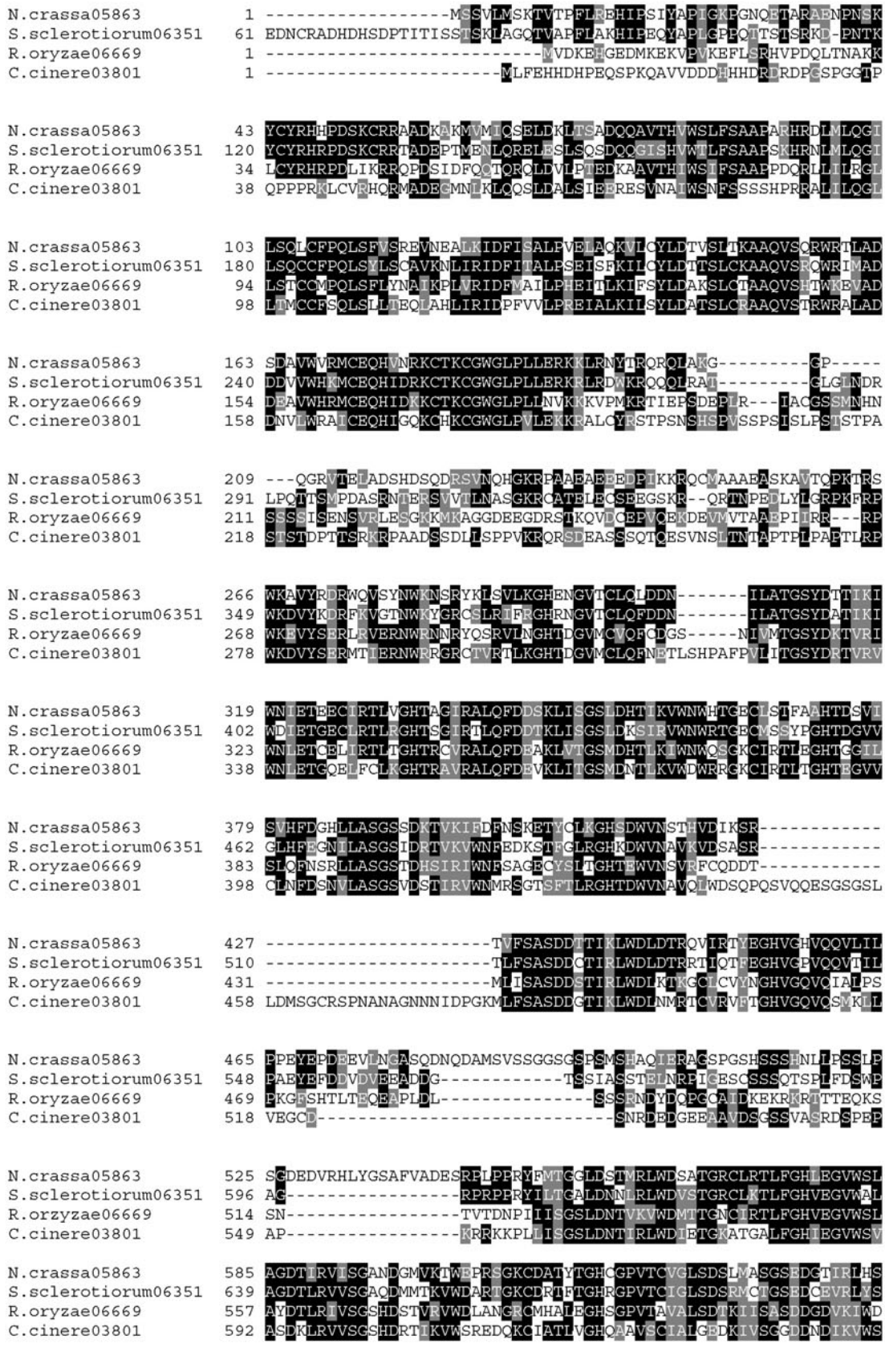


FIGURE 2 Schematic diagram of the *Neurospora crassa* sulfur regulatory system. (A) Sulfur-limited conditions. Plus symbols represent a positive effect. (B) Sulfur-sufficient conditions. The bound SCON-2-CYS-3 condition and subsequent proteolysis of CYS-3 represent the action of the SCF^{SCON2} complex.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R.oryzae06669 1 ----------MVDKEHGEDMKEKVPVKEFLSRHVPDQLTNAKK
C.cinere03801 1 ----------MLFEHHDHPEQSPKQAVVDDDHHDRDRDPGSPGGTP
N.crassa05863 43 YCYRHHPDSKCRRAADKAKMVMIQSELDKLTSADQQAVTHVWSLFSAAEARHRDLMLQGI
S.sclerotiorum06351 120 YCYRHRPDSKCRRTADEPTMENLQRELESLSQSDQQGISHVWTLFSAAESKHRNLMLQGI
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N.crassa05863 645 FKPCRQ----------
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R.oryzae06669 617 YGV----------
C.cinere03801 652 FA----------

et al., 1998), the F-box protein, which corresponds to *N. crassa* SCON-2, and *S. cerevisiae* Met30p. In contrast to the highly regulated expression of *scon-2*$^{+}$, *sconB* expression increases only twofold under sulfur-limited growth conditions. SconC (Piotrowska et al., 2000), the Skp1p (yeast) homolog, corresponds to SCON-3 of *N. crassa* and is expressed similarly. The nature of the seemingly fundamental difference in the respective control mechanisms used to regulate metR and *cys-3*$^{+}$ has not been resolved to date.

Storage and Processing

Choline-*O*-sulfate is a significant storage form of sulfur in fungi. Examples include (i) choline-*O*-sulfate usage during germination of *N. crassa* for the production of sulfate (McGuire and Marzluf, 1974; Marzluf, 1997); and (ii) in *P. brasiliensis*, choline sulfatase (at least at the mRNA level) is sharply upregulated in the mycelia to yeast phase transition suggesting mobilization of internal sulfur stores (Ferreira et al., 2006). Further, *N. crassa* can take up exogenous choline-*O*-sulfate by a specific permease and either store or use choline sulfatase to release the sulfate for assimilation. Orthologs to the *N. crassa* choline sulfatase (NCU08364) are observed in a wide range of fungi (e.g., *U. maydis*, *F. oxysporum*, and *H. capsulatum*) but are notably absent in *B. dendrobatidis* (http://www.broad.mit.edu/cgi-bin/annotation/fgi/blast_page.cgi) (Paietta, unpublished).

Figure 1 demonstrates the means by which excess sulfur can be routed into choline-*O*-sulfate. The enzyme PAPS-choline sulfotransferase can transfer the sulfate group from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to choline, generating choline-*O*-sulfate. Sulfation of other organic compounds (as with choline) might serve as a route of assimilation and storage but has not been studied in detail to date.

The sulfate acquired by multiple routes funnels into the sulfur assimilatory pathway eventually leading to the synthesis of cysteine (Fig. 1). The pathway operates as follows: (i) using sulfate, ATP sulfurylase produces adenosine-5′-phosphosulfate (APS); (ii) adenylyl sulfate kinase produces PAPS; (iii) PAPS reductase generates sulfite; (iv) sulfite reductase converts sulfite into sulfide; and last, (v) cysteine synthase uses sulfide and *O*-acetyl serine for the synthesis of cysteine. The *N. crassa* enzymes corresponding to each step in the pathway are shown in Fig. 1. A useful summary of *N. crassa* pathways and identified gene loci is available (Radford, 2004). Similarly, recent genomic analysis of *U. maydis* identified genes encoding the enzymes for each step in the pathway (McCann and Snetselaar, 2008).

Analysis of the *N. crassa* (Borkovich et al., 2004) and *U. maydis* (McCann and Snetselaar, 2008) genomic data reveals every needed step for the interconversion of methionine and for the *S*-adenosylmethionine cycle. The pathway (using corresponding *N. crassa* genes) begins with cystathionine γ-synthetase (NCU08117, NCU05093, and NCU02430) producing cystathionine from cysteine and *O*-acetyl homoserine. Cystathionine β-lyase (NCU07987) then cleaves cystathionine to generate homocysteine. Methionine is then produced from homocysteine and 5-methyl tetrahydrofolate by methionine synthase (NCU06512, NCU08434, and NCU10020). SAM synthetase (NCU06512) generates AdoMet (SAM) from methionine and ATP, and the AdoMet is used in a variety of methyltransferase reactions. After donating a methyl group, SAM is converted to *S*-adenosylhomocysteine and homocysteine (to be recycled into methionine). As far as the internal metabolism of sulfur is concerned, an interesting aspect of sulfur metabolism notable in the fungi is the presence of a complete set of enzymes to go back and forth from cystathionine to homocysteine (and back to methionine) and from cystathionine to cysteine. Most eukaryotes have one or the other capability (for example, human metabolism can only proceed from cystathionine to cysteine). The enzymes cystathionine γ-synthase, cystathionine γ-lyase, cystathionine β-lyase, and cystathionine β-synthase are involved in this versatile pathway. Reverse transsulfuration has been maintained in the fungi with a widespread distribution. Figure 4 shows a sequence alignment of the key transsulfuration enzyme cystathionine γ-lyase. Note cystathionine γ-lyase orthologs in fungi ranging from chytrids through Zygomycetes.

PHOSPHORUS

Acquisition of Phosphorus: Phosphorus Sources

Phosphorus is an essential macroelement with a relatively high cellular content (inferior only to carbon and nitrogen). Phosphorus is a component of, or needed for, the synthesis of numerous key biomolecules; including nucleic acids, phospholipids, and cellular metabolites. Further needs for phosphorus include energy metabolism and widespread cellular control mechanisms. Fungal cells, therefore, have substantial demands for phosphorus and correspondingly complex acquisition strategies. The particular focus here is on the regulated aspects of phosphorus acquisition.

Phosphorus as inorganic phosphate (P_i) is readily accessed by specialized transporters. These phosphate transporters must operate at micromolar concentrations; for example, soil solution levels of P_i are seldom above 10 μM (Bieleski, 1973). Another factor is the form of P_i that is present at various environmental pHs; for example, at pH values of <6.0, it exists primarily as $H_2PO_4^-$ available for uptake. Other forms of inorganic phosphates include pyrophosphate (PP_i) and polyphosphate ($polyP_i$). The phosphate from PP_i and $polyP_i$ is accessible through the action of, for example, alkaline phosphatases.

Other sources of phosphate include the organophosphates (e.g., phosphorylethanolamine) (Beever and Burns, 1980). Besides hydrolysis of these compounds by secreted enzymes, certain organophosphates can be transported into the cell and then utilized. For example, *N. crassa* has a repressible phosphorylethanolamine-phosphorylcholine permease (Metzenberg, 1979). When grown under phosphate-limiting conditions, the expression of this organophosphate permease markedly increases.

Further, fungi commonly produce a variety of enzymes to access phosphorus from nucleic acids. Examples, again from *N. crassa*, include regulated production of a 5′-nucleotidase

FIGURE 3 *N. crassa* F-box regulator SCON-2 sequence alignment with a representative selection of fungal proteins showing homology. Species represented, from top to bottom, are *N. crassa*, *Sclerotinia sclerotiorum*, *R. oryzae*, and *C. cinereus*. Numerical designations represent the gene identifiers in the Fungal Genome Initiative database at http://www.broad.mit.edu. Identical residues are shown as white on black, while similar residues are shown as white on gray.

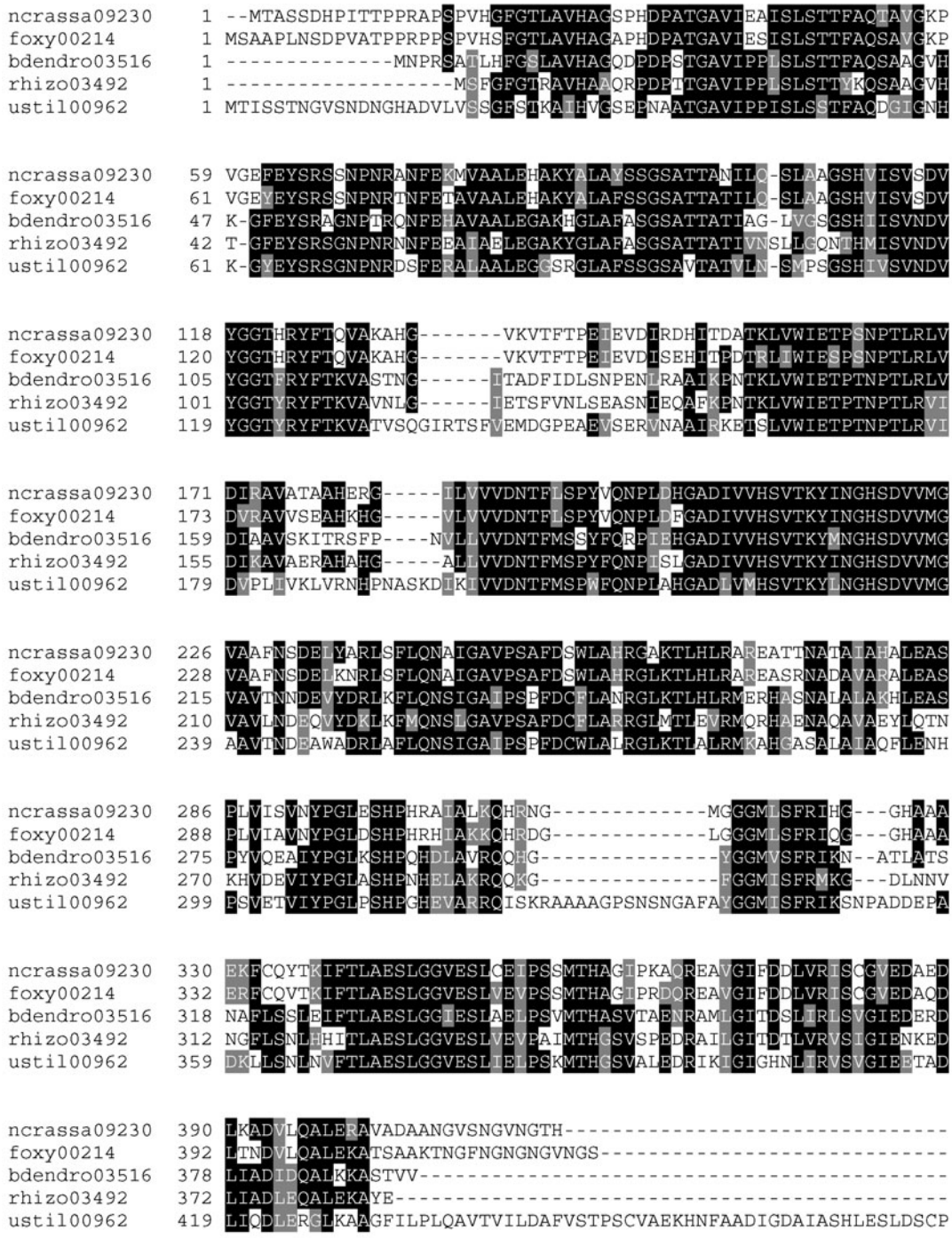

FIGURE 4 *N. crassa* cystathionine γ-lyase sequence alignment with a representative selection of fungal proteins showing homology. Species represented, from top to bottom, are *N. crassa*, *F. oxysporum*, *B. dendrobatidis*, *R. oryzae*, and *U. maydis*. Numerical designations represent the gene identifiers in the Fungal Genome Initiative database at http://www.broad.mit.edu. Identical residues are shown as white on black, while similar residues are shown as white on gray.

(Hasunuma and Ishikawa, 1977) and at least three nucleases (Hasunuma,1973). Similar work in *A. nidulans* has identified a repressible secreted nuclease (Kafer et al., 1989). Extracellular nucleases have been characterized in a few other fungi (e.g., *Rhizopus stolonifer*) (Rangarajan and Shankar, 1999). Interestingly, mutant hunts using culture medium containing DNA with detection based on "nuclease halo" or filtration techniques have been used with *A. nidulans*, *N. crassa*, and *U. maydis* to obtain nuclease-deficient strains (Kafer and Fraser, 1979; Kafer et al., 1989; Badman, 1972).

Fungi may encounter many sources of nucleic acids in the environment (e.g., dead plant cells) to exploit for needed phosphate. A representative sequence of phosphate acquisition in *N. crassa* would be the initial hydrolysis of the nucleic acids by phosphate-repressible nucleases, followed by hydrolysis of the resulting esters by the repressible acid phosphatase, 5′-nucleotidase, and repressible alkaline phosphatase and subsequently transport of the released phosphate into the cell (Metzenberg, 1979).

Note that regulation related to pH sensing, which is relevant to phosphorus metabolism, is covered in chapter 31 (this volume). Briefly, many fungi can operate under a wide range of pH values and regulate the production of certain proteins (e.g., transporters and secreted enzymes) appropriate to the ambient pH (Penalva and Arst, 2002; Arst and Tilburn, 2004). For example, in *A. nidulans*, secretable alkaline phosphatase (PalD) level increases 40-fold as the external pH is increased from 5 to 8 (Caddick and Arst, 1986). An acid phosphodiesterase is also known to be similarly pH responsive in *A. nidulans* (Caddick et al., 1986). In contrast, acid phosphatase levels in *A. niger* show the opposite pattern (MacRae et al., 1988) and in *A. nidulans* (PacA) (Caddick et al., 1986). pH regulation proceeds through action of the PacC zinc finger transcription factor and the associated regulatory pathway which appears to be conserved throughout the Ascomycetes (Penalva and Arst, 2002).

Acquisition of Phosphorus: Transport

Inorganic phosphate is essential for numerous cellular reactions, which often utilize millimolar levels of phosphate. In order to concentrate available phosphate present in the environment (often at a micromolar level), fungal cells use a set of specialized phosphate transporters. During growth on limiting phosphate, fungal cells respond by increasing the capacity for phosphate uptake. *N. crassa* serves as the filamentous fungal model system for discussion here, but conservation of phosphate transporters is observed throughout the fungi.

N. crassa has a combination of low-affinity and high-affinity phosphate transport systems. In contrast to yeast, with four transporters (PHO84, PHO89, PHO90, and PHO91) (Persson et al., 2003), *N. crassa* appears to have a total of three transporters. A low-affinity transporter (termed System I) in *N. crassa* whose level is not phosphate starvation responsive has been described (Lowendorf and Slayman, 1975). Under culture conditions of phosphate sufficiency this transporter is sufficient to supply needed levels of phosphate. A high-affinity transporter (termed System II [Lowendorf and Slayman, 1975; Lowendorf et al., 1975]) is derepressed upon phosphate limitation. The System II transporter is encoded by the *pho-4*$^+$ gene (Mann et al., 1989). The PHO-4 transporter has been characterized as an Na^+-phosphate symporter (Versaw and Metzenberg, 1995). For PHO-4, phosphate uptake was found to be stimulated substantially (85-fold) by Na^+. Typically, Na^+-coupled symporters are also associated with animal systems (Versaw and Metzenberg, 1995). The PHO-4 transporter corresponds to Pho89p of *S. cerevisiae* (Bun-ya et al., 1991). Essentially universal conservation of this Na^+ symporter is seen throughout the fungi based on homology searches (Paietta, unpublished). An additional high-affinity phosphate transporter, PHO-5, is also present in *N. crassa* (Versaw, 1995) and is derepressible following phosphate limitation. PHO-4 and PHO-5 together make up the high-affinity phosphate transport system of *N. crassa* based on a lack of growth of a *pho-4 pho-5* null double mutant under phosphate-restrictive conditions (Versaw, 1995). PHO-5 represents a H^+-phosphate symporter (Versaw and Metzenberg, 1995), in that PHO-5-mediated transport is sensitive to an elevated pH. PHO-5 corresponds to the *S. cerevisiae* Pho84p (Martinez and Persson, 1998). Again, striking conservation of this H^+-phosphate symporter is seen in a broad range of fungi (Fig. 5). Of note is that the chytrid *B. dendrobatidis* possesses two PHO-5 homologs. Interestingly, a chytrid, *Rhizophydium planktonicum*, appears to be able to outgrow its host under conditions of phosphorus limitation (Bruning, 1991). A final point regarding uptake is that, as mentioned above for sulfur, mycorrhizal fungi can enhance the uptake of phosphorus by plants (Bolan, 1991).

Response to Phosphorus Limitation and Regulation of Acquisition

The most detailed knowledge of phosphorus metabolism in filamentous fungi has been obtained in studies of *N. crassa* and *A. nidulans*. Early studies by Metzenberg established the outline of the regulatory system (summarized by Davis, 2000), and it is satisfying to see the fit of the original epistatic and other studies to the molecular mechanism.

When *N. crassa* is grown on low levels of inorganic phosphate (or organophosphorus), a set of eight proteins involved in various aspects of phosphorus acquisition are known to be derepressed. Included are an alkaline phosphatase, 5′-nucleotidase, several nucleases, two phosphate transporters, and a phosphorylethanolamine-phosphorylcholine permease (Metzenberg, 1979), which have been discussed regarding their acquisition functions above. The *N. crassa* regulated gene set is likely incomplete, since in *S. cerevisiae* at least 22 genes are derepressed in response to P_i limitation as determined by microarray analysis (Ogawa et al., 2000). The *N. crassa* phosphate-related genes are controlled by a complex regulatory system made up of the NUC-1, NUC-2, PREG, and PGOV proteins (equivalent to Pho4p/Pho2p, Pho81p, Pho80p, and Pho85p, from *S. cerevisiae*, respectively).

In *N. crassa*, NUC-1 is the DNA-binding transcriptional activator in the phosphorus regulatory system. NUC-1 contains a basic helix-loop-helix motif and regulatory domain (Kang, 1993; Peleg and Metzenberg, 1994). By comparison, the *S. cerevisiae* equivalent to NUC-1 appears to be the DNA-binding transcriptional activators Pho2p and Pho4p (Persson et al., 2003). The action of NUC-1 is regulated by a kinase complex (the role of phosphorylation has yet to be rigorously verified). The cyclin PREG, cyclin-dependent kinase PGOV, and CDK inhibitor NUC-2 constitute key components in the control system (Fig. 6). Under high-phosphate conditions, NUC-1 is localized to the cytoplasm and presumably phosphorylated to some degree (with analogy to Pho4p phosphorylation observed in the yeast system [Kaffman et al., 1994]) or sequestered in a complex (Peleg et al., 1996a, 1996b). When the cells are limited for phosphate, NUC-2 acts to inhibit the kinase complex, and unphosphorylated NUC-1 (or NUC-1 released from sequestration) accumulates in the nucleus and activates transcription of a battery of phosphorus metabolism genes. At intermediate phosphate levels it seems likely the complex would be partially active. A recent microarray analysis of the *N. crassa* system using a *nuc-2* strain has identified genes involved in mRNA translation as being specifically up-regulated (Gras et al., 2007).

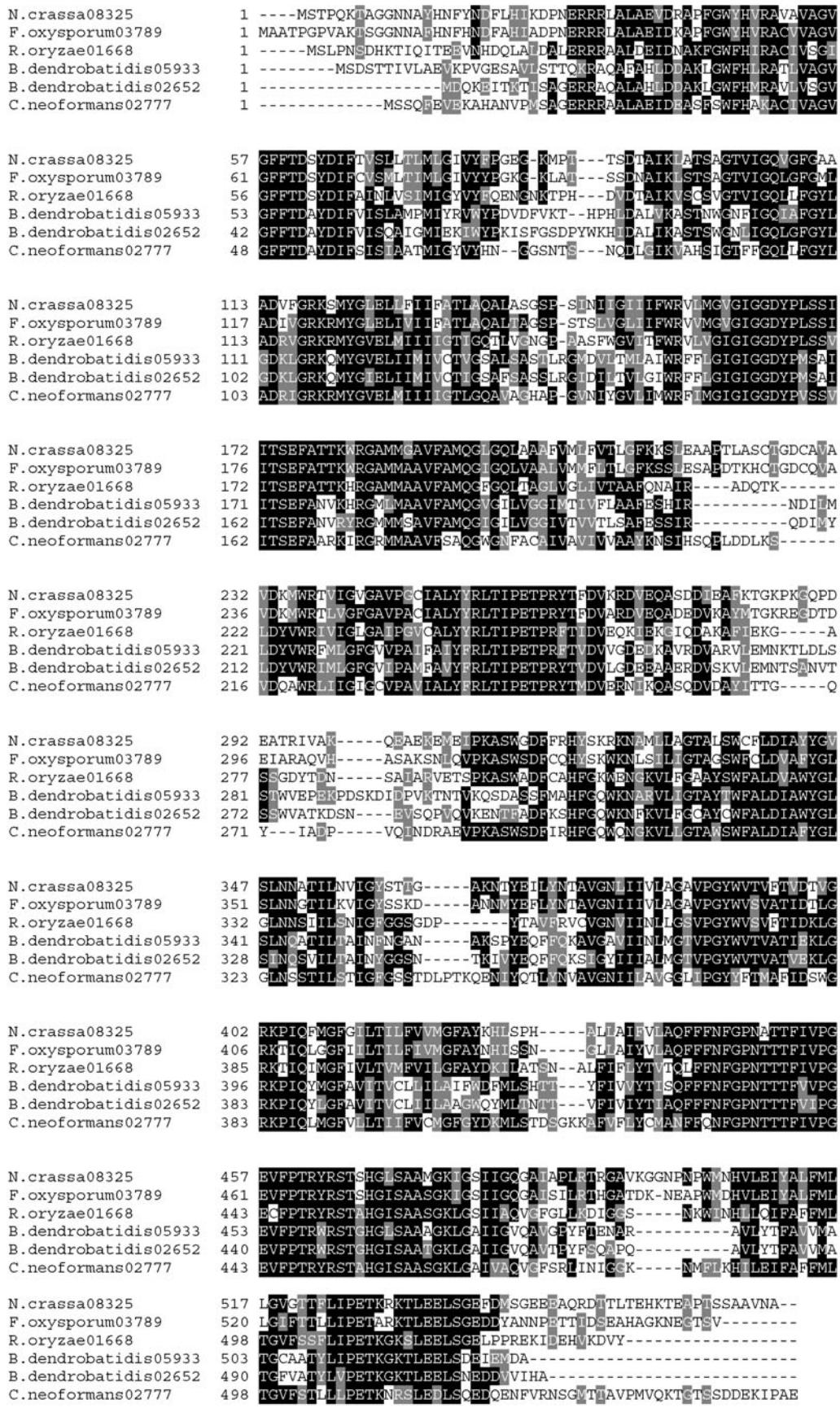

FIGURE 5 *N. crassa* phosphate transporter *PHO-5* sequence alignment with a representative selection of fungal proteins showing homology. Species represented, from top to bottom, are *N, crassa*, *F. oxysporum*, *R. oryzae*, *B. dendrobatidis*, and *C. neoformans*. Numerical designations represent the gene identifiers in the Fungal Genome Initiative database at http://www.broad.mit.edu. Identical residues are shown as white on black, while similar residues are shown as white on gray.

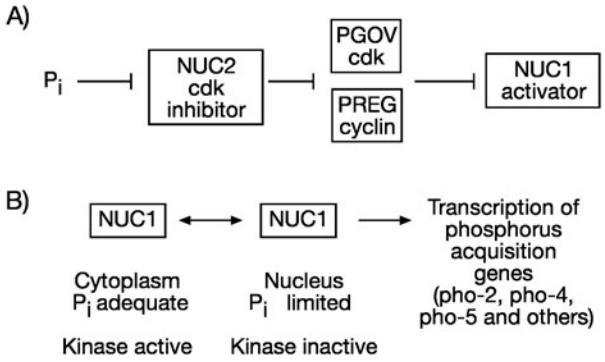

FIGURE 6 Schematic diagram of the *N. crassa* phosphorus regulatory system. (A) Arrangement of pathway components demonstrating negative effect of P_i. (B) Model for NUC-1 translocation to the nucleus and subsequent activation of phosphorus acquisition gene expression.

As discussed above, overall, the *N. crassa* and *S. cerevisiae* control systems closely parallel each other. In Fig. 7 the PGOV regulator of *N. crassa* is shown aligned with orthologs from a diversity of fungal species (*F. oxysporum*, *R. oryzae*, *B. dendrobatidis*, and *C. cinereus*), further demonstrating putative regulatory similarities. Detailed molecular work has identified an apparent difference in the *A. nidulans* system. In *A. nidulans*, PhoA and PhoB appear to represent two homologs of PGOV. PhoA does not regulate the classic system, but instead modulates development in a phosphate-dependent manner (Dou et al., 2003; Wu et al., 2004), while deletion of *phoB* does not lead to an obvious phenotype. The phenotype of the *phoA phoB* double mutant, which is lethal, suggests involvement in cell cycle control and morphogenesis (Dou et al., 2003). The *phoA phoB* genes (as a pair) are essential, as is the case for PGOV in *N. crassa* (NCU07580,http://www.dartmouth.edu/~neurosporagenome/knockouts_completed.html), but the equivalent gene (pho85) in *S. cerevisiae* is not essential (Dou et al., 2003). Currently it is not clear if there may be another protein playing the role of PGOV in *A. nidulans*. The results are suggestive of dual roles for proteins, and caution on assigning functional roles for proteins based on homology should be exercised.

Another level of regulation is suggested by work with *N. crassa pho-2* (alkaline phosphatase) (Han et al., 1987; Nozawa et al., 2002) in that culture pH does not affect gene expression; however, the enzyme is only substantially secreted under alkaline conditions and this may be dependent on the glycosylation of the enzyme. On yet another level, positive- and negative-feedback loops in the *S. cerevisiae* PHO pathway allow precise adjustment in the complement of high- and low-affinity phosphate transporters (Wykoff et al., 2007). These studies indicate that the phosphorus

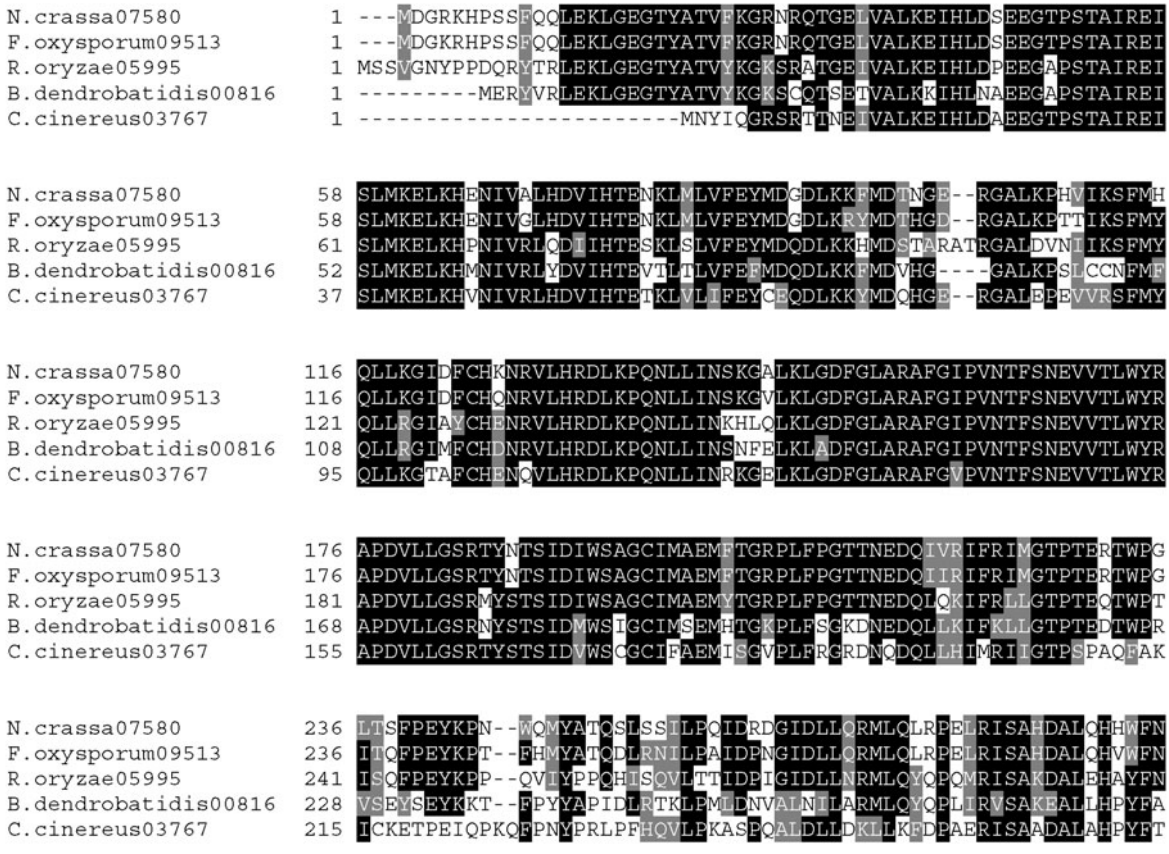

FIGURE 7 *N. crassa* regulator PGOV sequence alignment with a representative selection of fungal proteins showing homology. Species represented, from top to bottom, are *N. crassa*, *F. oxysporum*, *R. oryzae*, *B. dendrobatidis*, and *C. neoformans*. Numerical designations represent the gene identifiers in the Fungal Genome Initiative database at http://www.broad.mit.edu. Identical residues are shown as white on black, while similar residues are shown as white on gray.

acquisition system should continue to reveal new strategies for optimization of nutrient acquisition.

Storage

Maintaining a stable cellular concentration of P_i is necessary for numerous enzymatic reactions. Internal P_i homeostasis is accomplished by membrane transport and exchange between intracellular pools of P_i, the details of which are beyond the current consideration of phosphorus acquisition. One aspect of P_i homeostasis involves the role of polyP [i.e., poly$(P)_3$ to poly$(P)_{N+3}$], which is accumulated by many eukaryotes, including fungi (Harold, 1966). Many diverse cellular roles have been suggested for polyP including the storage of P_i (Kulaev and Kulakovskay, 2000; Kulaev and Vagabov, 1983). Vacuolar storage of polyPs in *N. crassa* has been demonstrated where most (at least 78%) of the polyP pool is localized as such (Cramer et al., 1980). Both *N. crassa* and yeast are known to accumulate polyP after being switched from phosphate-limiting to high-phosphate medium and to utilize this reservoir upon P_i limitation (Harold, 1962; Kulaev and Kulakovskay, 2000). These findings suggest a potentially important role in P_i homeostasis and possible involvement with the phosphorus regulatory system. An intriguing recent finding in *U. maydis* has indicated a role for protein kinase A-regulated polyP accumulation in controlling morphogenesis and virulence (Boyce et al., 2006).

IRON

Acquisition of Iron: Sources and Transport

Iron is a crucial nutritional microelement used in numerous cellular reactions ranging from electron transport to the synthesis of deoxyribonucleotides. Although iron is abundant in the environment and only low levels are required for optimal cellular function, this belies its generally low bioavailability in an aerobic environment. Iron can exist in the oxidized ferric (Fe^{3+}) or ferrous (Fe^{2+}) forms and demonstrates a reduced bioavailability because it is generally found in an oxidized ferric state of low solubility (e.g., oxyhydroxide particles). Filamentous fungi use a versatile combination of strategies to obtain sufficient amounts of this frequently limiting nutrient. In addition, the fungi carefully regulate iron uptake and storage, since excess iron can have detrimental effects through generation of reactive oxygen species (Halliwell and Gutteridge, 1992). A number of recent reviews provide a detailed look at various aspects of iron metabolism (Haas, 2004; Johnson, 2008; Miethke and Marahiel, 2007; Philpott, 2006; Philpott and Protchenko, 2008), including fungal-genome-based comparisons (Haas et al., 2008). Presented here is an overview of acquisition, storage, and regulation. Although *S. cerevisiae* has been the most extensively studied system, differences in comparison to most filamentous fungi exist in terms of the lack of siderophore synthesis in yeast and overall control of iron gene regulation (i.e., Aft1p transcriptional activation in yeast versus GATA factor transcriptional repression in filamentous fungi).

Two general means of iron acquisition appear to have a widespread distribution among the fungi. The first is a mechanism using the extracellular reduction of ferric to ferrous iron (by metalloreductases) followed by uptake through a high-affinity system (i.e., a ferroxidase and iron permease) or, alternately, under high-iron conditions the low-affinity direct uptake of the resulting ferrous iron. The second is the synthesis, secretion, and subsequent transport into the cell of siderophore-Fe^{3+} complexes (or extracellular siderophore reduction and direct ion transport). Note that although species such as *S. cerevisiae* do not synthesize siderophores, they do produce an array of siderophore transporters to utilize siderophores secreted by other organisms for iron acquisition.

S. cerevisiae has served as the primary model system for studies of extracellular Fe^{3+} reduction by a series of iron-regulated metalloreductases. Note that iron salts and iron chelates (e.g., compounds such as ferric citrate, as well as iron-siderophore complexes) can serve as substrates for the metalloreductases. By action of the metalloreductases, the iron can be released from siderophores at the cell surface and taken up through the ferroxidase/iron permease complex (see below). In yeast, there is some specialization of function among the Fre1p, Fre2p, Fre3p, and Fre4p metalloreductases. For example, Fre1p carries out the reduction of iron salts and chelates such as ferric citrate (Dancis et al., 1992). While all of the Fre proteins appear to be involved in release of iron from siderophores, they each do have characteristic ranges of siderophore substrate specificities (i.e., hydroxamates and catecholates for Fre1p and Fre2p; hydroxamates for Fre3p; and rhodotorulic acid for Fre4p) (see Philpott, 2006). The presence of putative metalloreductases among the filamentous fungi appears to be widespread (Haas et al., 2008).

Again using *S. cerevisiae* as a model, the Fet3p/Ftr1p ferroxidase/iron permease complex serves as a high-affinity iron acquisition system. The Fe^{2+} generated by the metalloreductases (e.g., Fre1p) is oxidized to Fe^{3+} by the Fet3p ferroxidase, and the Fe^{3+} produced is brought into the cell by the Ftr1p transporter (Askwith et al., 1994; De Silva et al., 1995; Stearman et al., 1996). The rationale for the oxidase/permease combination and coupled changes in iron oxidation state is not understood at present. The oxidase/permease high-affinity system appears to be a common fungal strategy for iron acquisition based on the finding of Ftr1p homologs in many species (Haas et al., 2008). Fungal exceptions in not having proteins homologous to Ftr1p appear to be *A. nidulans* and *C. cinereus* (Hass et al., 2008). A few examples of species (in addition to *S. cerevisiae)* in which a functional oxidase/permease system has been confirmed include *U. maydis* (Eichhorn et al., 2006), *Fusarium graminearum* (Park et al., 2007), and *A. fumigatus* (Schrettl et al., 2004). The Fe^{3+} transported into the cell may then commonly, at least in the filamentous fungi, be complexed with an intracellular siderophore for storage (or be reduced and utilized). Studies with *U. maydis* demonstrating the necessity of the ferroxidase/permease for virulence (Eichhorn et al., 2006) emphasize a common theme of the importance of iron acquisition for fungal pathogenicity.

Either metalloreductase-generated or existing ferrous iron can also be taken up directly by a low-affinity system (i.e., a ferrous transporter) in a situation where iron levels are not limiting. Examples include Fet4p in *S. cerevisiae* (K_m of 30 μM for Fe^{2+}), which also has low specificity (copper, zinc, and cadmium are also transported) (Hassett et al., 2000; Dix et al., 1994; Eide et al., 1992), and the Nramp family of divalent metal transporters (Chen et al., 1999). Haas (2004) suggests that low-affinity transporters may be conserved (i.e., FET4 orthologs are present in *S. pombe*, *N. crassa*, and *A. fumigatus*).

Siderophore-mediated nonreductive iron uptake represents the second type of high-affinity system for iron

acquisition. Siderophores are synthesized under iron-limiting conditions to chelate ferric (Fe^{3+}) iron. The siderophores can be classified as catecholates, phenolates, carboxylates, hydroxylates, and mixed types (Mietke and Marahiel, 2007). The filamentous fungi primarily produce hydroxamate-type siderophores, using ornithine as a precursor. Within the hydroxamate siderophores, four structural subgroups have been described: (i) coprogens, (ii) ferrichromes, (iii) fusarines, and (iv) rhodotorulic acid. Only within the Zygomycetes is a carboxylate-type siderophore, rhizoferrin, synthesized (Thieken and Winkelmann, 1992). Note that *S. cerevisiae*, *Candida albicans*, and *C. neoformans* do not synthesize siderophores (although they can obtain iron by uptake of siderophores derived from other organisms) (Haas et al., 2008; Winkelmann, 2007). Details of the synthesis pathways are beyond the scope of this chapter, but they are highly regulated. For example, in *U. maydis* the first step of siderophore synthesis is catalyzed by L-ornithine N^5-oxygenase encoded by the *sid1* gene. The Urbs1 iron-responsive GATA-factor negatively regulates siderophore production by repression of the *sid1* gene (An et al., 1997a). The importance of siderophore synthesis in pathogenicity is underscored by work with *H. capsulatum* where SID1 (L-ornithine N^5-oxygenase)-deficient strains demonstrate an in vivo growth defect and impaired host colonization (Hwang et al., 2008). A similar effect on virulence with regard to the equivalent gene, *sidA*, is seen with *A. fumigatus* (Schrettl et al., 2004).

Filamentous fungi often produce more than one siderophore, having different secreted extracellular versus intracellular forms (Winkelmann, 2007; Haas et al., 2008). In a number of fungi, intracellular iron is stored bound to siderophores that are not secreted and the secreted siderophores specialize in obtaining iron from the environment. For example, *A. nidulans* secretes fusarinine-type siderophores (triacetylfusarinine C and fusarinine C), while producing ferricrocin for intracellular iron storage. *N. crassa* secretes coprogen and as with *A. nidulans* uses ferricrocin primarily intracellularly. *U. maydis* secretes ferrichrome and ferrichrome A, while also using ferrichrome for iron storage. Each fungal species has a distinct profile of hydroxamate siderophore subtypes.

After secretion, the siderophore chelates ferric (Fe^{3+}) iron and the complex is available for uptake. The uptake typically involves the entire Fe^{3+}-siderophore complex being transported into the cell (or alternately extracellular reduction of the siderophore occurs with direct permease uptake). Siderophores are transported by the ARN/SIT (UMF/SIT) subfamily of the major facilitator superfamily (Pao et al., 1998; Winkelmann, 2001, 2007). Although *S. cerevisiae* does not synthesize siderophores, it produces four siderophore transporters with different siderophore specificities (Sitp/Arn3p, Arn1p, Taf1p/Arn2p, and Enb1p/Arn4p). These transporters have varying degrees of specificity for various types of siderophores (Johnson, 2008; Philpott, 2006). For example, Arn3p/Sitp can transport ferrichromes, coprogen, and bacterial ferrioxamines, while Arn2p/Taf1p recognizes triacetylfusarinine C. In comparison, a group of SIT family transporters has been reported in *A. nidulans* and designated MirA, MirB, and MirC (Haas et al., 2003). MirB, for example, transports triacetylfusarinine C. Overall, *A. nidulans* appears to encode at least 10 siderophore transporters based on homology searches (Haas et al., 2008) and has an expected capability to transport a range of siderophore types.

The siderophore transporters have been a particular research focus because they represent a protein family that is unique to fungi (Hsaing and Baillie, 2005). Thus, by exploiting this phylogenetic difference, antifungal agents might be delivered by a "Trojan horse" approach, where attachment of the agent to the siderophore is performed with subsequent intracellular delivery by the specific siderophore transporter (Roosenberg et al., 2000; for a bacterial example, see Mollman et al., 2009).

A final consideration is in regard to the acquisition of host iron sources by fungal pathogens. *C. albicans* can switch between yeast, pseudohyphal, and hyphal forms and provides an example of iron acquisition from certain host proteins. *C. albicans* can use host heme (as hemin) by transport and subsequent degradation by heme oxygenase (Santos et al., 2003; Weismann and Kornitzer, 2004). *C. albicans* can also use iron-loaded transferrin (via metalloreductases) as an iron source (Knight et al., 2005). The dimorphic fungus *H. capsulatum* presents another example of a fungus that can use heme (as hemin) as a sole iron source in culture (Foster, 2002).

Response to Iron Limitation and Regulation of Acquisition

Fungi tightly regulate iron uptake, use, and storage to maintain a balance between an adequate supply of iron and the toxic effects of iron excess. Given that no fungal iron excretory mechanism is present, the regulation of iron acquisition becomes a crucial point of control. In *S. cerevisiae*, the Aft1p and Aft2p transcription factors are primarily involved in the regulation of iron-responsive genes (Philpott and Protchenko, 2008). In other fungi, iron-regulated gene expression is primarily controlled by GATA-type factors that repress the transcription of target genes. While the *S. cerevisiae* Aft proteins are transcriptional activators and other fungi use GATA transcriptional repressors, in both systems the common end point is that the iron acquisition genes (and other target genes) are expressed under conditions of iron limitation. Regulation in the iron-responsive GATA-factor-controlled systems of *U. maydis*, *A. nidulans*, *C. albicans*, and *N. crassa* provides clear examples for filamentous fungi. Note that Aft homologs have not been identified in the filamentous fungi to date (Haas et al., 2008).

Iron regulation in most fungi proceeds through the action of GATA transcription factors. Initial work on an iron-responsive GATA factor was carried out with *U. maydis* and Urbs1 (Voisard et al., 1993). Additional examples include the iron-responsive GATA factors SreA in *A. nidulans* (Haas et al., 1999), SRE in *N. crassa* (Zhou et al., 1998), and Sfu1p in *C. albicans* (Lan et al., 2004). A 5′-GATA-3′ element is the consensus binding site for the zinc finger GATA factors, which are involved, aside from iron control systems, in the regulation of a number of metabolic and developmental systems (Scazzocchio, 2000). Unlike other fungal GATA factors, the iron-responsive GATA subgroup has two zinc finger motifs separated by a central cysteine-rich region. Figure 8 shows an alignment of typical fungal GATA factors. In the case of the *N. crassa* SRE GATA factor, both zinc fingers are needed for normal regulatory function (Zhou and Marzluf, 1999), while in *U. maydis* Urbs1 the C-terminal zinc finger is sufficient for target gene repression (An et al., 1997b). As with *U. maydis*, deletion of *sre* in *N. crassa* and *sreA* in *A. nidulans* results in derepression of target genes (Zhou et al., 1998; Haas et al., 1999). These studies also show that the GATA factor genes

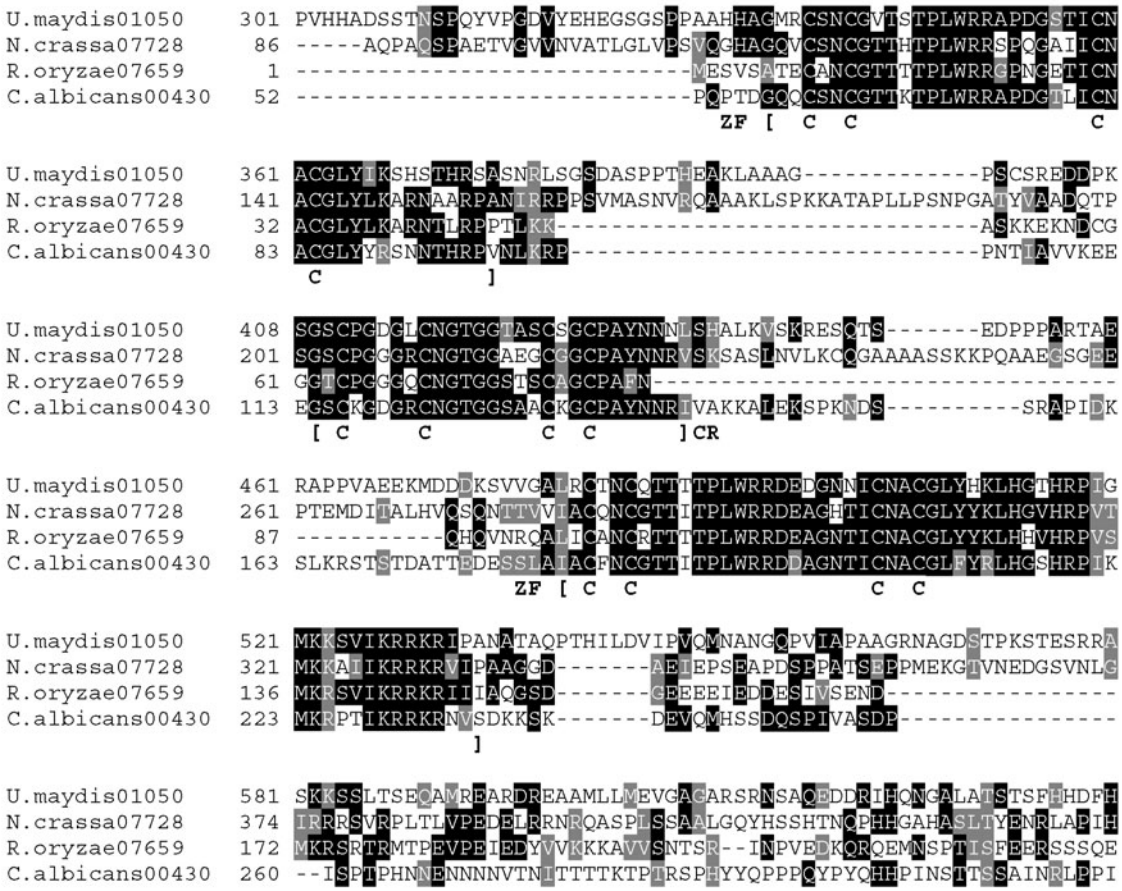

FIGURE 8 *U. maydis* iron-responsive GATA-factor Urbs1 sequence alignment with a representative selection of fungal proteins showing homology. Species represented, from top to bottom, are *U. maydis*, *N. crassa* (SRE), *R. oryzae* (RO3G 07659), and *C. albicans* (Sfu1p). Numerical designations represent the gene identifiers in the Fungal Genome Initiative database at http://www.broad.mit.edu. Sequences are truncated to compare only zinc fingers and central conserved cysteine-rich region as defined by brackets with conserved cysteine residues indicated (ZF, zinc finger; CR, cysteine-rich region). Identical residues are shown as white on black, while similar residues are shown as white on gray.

in *U. maydis* (*Urbs1*), *N. crassa* (*sre*), and *C. albicans* (*sfu1*) are constitutively expressed, while *A. nidulans sreA* shows regulated expression (down-regulation). The difference in GATA factor regulation suggests an additional level of control in the *A. nidulans* system.

Whereas in *U. maydis* the findings seem to be consistent with a role for Urbs1 as the sole regulatory factor involved in repression, a corepressor appears to be necessary in some other cases. Figure 9 shows the basic fungal GATA-factor iron-responsive control system but includes a corepressor (Tup1) as seen in *C. albicans* (Knight et al., 2005; Pelletier et al., 2007). In this model, the Sfu1p GATA transcription factor-Tup1 complex is targeted to the promoter binding site (i.e., the GATA sequence) under iron excess and presumably mediates repression by altering chromatin structure and inhibition of the transcriptional machinery (Gavin et al., 2000). When iron is limiting, the GATA factor is inactive and fails to repress the target genes through the Tup1 complex.

Additional complexity is seen in the *A. nidulans* control system, in which the expression of *sreA* itself is regulated. An elegant model that includes a role for HapX has been proposed (Hortschansky et al., 2007). The system includes regulated interactions between HapX, the CCAAT-binding core complex, and SreA. An interplay occurs whereby the presence of iron results in repression of *hapX* expression while the absence of iron results in the repression of *sreA* expression. Additionally, increasing levels of iron block (possibly directly) the repression by HapX of iron-consuming pathways (see Haas et al., 2008).

There is some evidence that these GATA factors may directly bind iron to mediate their iron-responsive system control. Typical of this work is the finding that recombinant purified SRE from *N. crassa* exhibits a reddish brown color with a typical iron binding spectrum (Harrison and Marzluf, 2002). Additionally, site-directed mutagenesis of the *N. crassa SRE* to replace the central cysteine residues results in a dominant repressor phenotype (i.e., iron unresponsive) (Harrison and Marzluf, 2002) and implicates those residues in iron binding.

As has been alluded to above, the iron-responsive GATA transcription factors control an array of genes involved in iron metabolism. The *S. cerevisiae* system has been the best characterized, and Aft1p activates a large set of genes involved in the uptake of iron from the environment

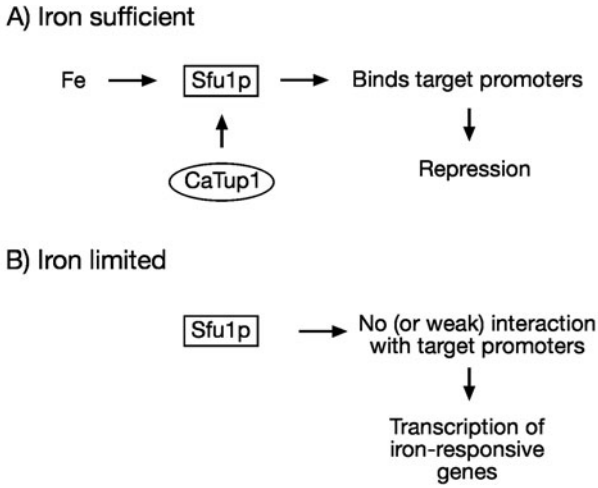

FIGURE 9 Schematic diagram of the *C. albicans* iron regulatory system. (A) Iron-sufficient (or iron excess) conditions. Sfu1p iron-responsive GATA factor interacts with corepressor Tup1 and represses the target gene. (B) Iron-limited conditions: no interaction of Sfu1p/Tup1 with target promoters and consequential derepression of gene expression.

(17 genes in total, encoding, e.g., metalloreductases, oxidase, and transporters [ferrous or siderophores]); efflux of iron from the vacuole (4 total); metabolic adaptation to iron (2 total); and a small group of other transporters (3 total) (summarized by Philpott and Protchenko, 2008; and Haas, 2004). In general, many of these components, if present, are also regulated by repression in the GATA factor iron regulons of filamentous fungi (see Haas, 2004, for a compilation). Additions to the yeast "base" regulon include the genes involved in siderophore synthesis, such as the *sidA* (L-ornithine N^5-monooxygenase), *sidB* (peptide synthetase), and *sidC* (peptide synthetase) genes of *A. nidulans*.

Storage of Iron

In many organisms (e.g., bacteria, plants, and animals) ferritin is used for iron storage. In contrast, the filamentous fungi (except for the Zygomycetes) use intracellular siderophores for this function. Environmental iron is chelated by extracellular siderophores and taken up by specific transporters or directly transported as ions (as discussed previously). Subsequently, the majority of iron is either directly channeled into metabolic use or stored using intracellular siderophores. As mentioned earlier, while *N. crassa* and *A. nidulans* secrete coprogen and triacetylfusarinine C, respectively, they use ferricrocin as an intracellular storage siderophore. In *N. crassa*, the acquired coprogen-bound iron would then be transferred to desiferricrocin. For example, the central role of ferricrocin for intracellular iron storage during mycelial growth in *A. nidulans* is indicated in that 5% of the iron pool is ferricrocin under iron limitation, but the proportion increases to 64% with an intracellular excess of iron (Eisendle et al., 2006). Iron storage is also an important factor in conidial germination. In *N. crassa*, conidia contain 47% of the iron pool as ferricrocin, but after germination and subsequent growth this iron pool is essentially depleted (Matzanke et al., 1987). Intracellular siderophores have been found to be important for development of *Cochliobolus heterostrophus* (Oide et al., 2007) and virulence of *M. grisea* (Hof et al., 2007).

Finally, it is worth noting that iron uptake and storage in the Zygomycetes appear to be exceptional in two regards. First, a polycarboxylate-type siderophore (rhizoferrin) is produced (Thieken and Winkelmann, 1992), and second, ferritin-like compounds (mycoferritin and zygoferritin) are used for iron storage (Matzanke, 1994). Many nonfungal species utilize this group of iron-rich proteins (i.e., ferritin, phytoferritins, and bacterioferritins) as storage molecules. Mycoferritin from *R. oryzae* demonstrates strong homology to mammalian ferritin (Fig. 10). In fact, two genes encoding conserved (94% identity) putative mycoferritins are found in *R. oryzae* (Fig. 10). It would be of interest to examine these forms as to their functional roles; perhaps hypothetically a spore versus mycelial distribution.

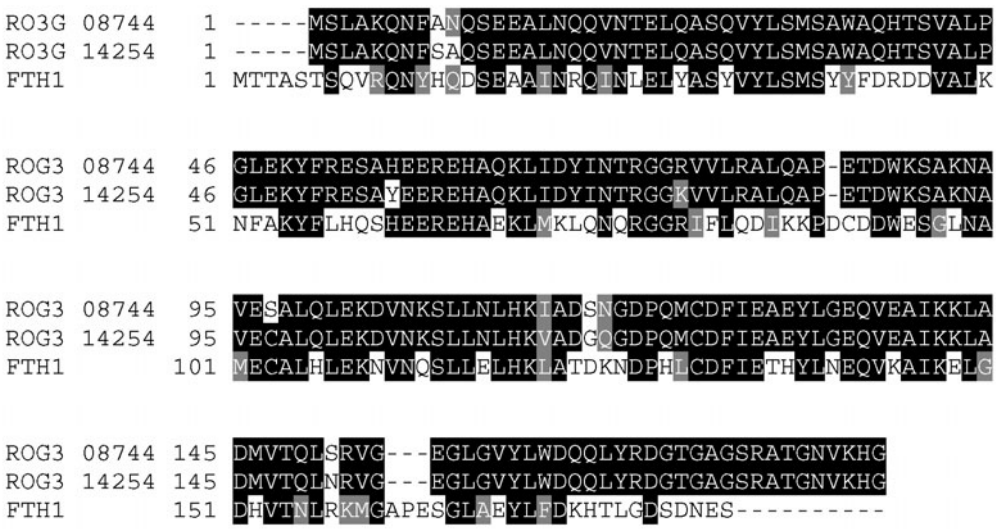

FIGURE 10 Sequence alignment of *R. oryzae* putative mycoferritins RO3G 08744 and RO3G 14254 with human ferritin (FTH1). Numerical designations for *R. oryzae* sequences represent the identifiers in the Fungal Genome Initiative database at http://www.broad.mit.edu. Identical residues are shown as white on black, while similar residues are shown as white on gray.

CONCLUDING REMARKS

The filamentous fungi have versatile, highly regulated systems for the acquisition of sulfur, phosphorus, and iron. Many useful mechanistic details are well understood for the regulatory systems involved; however, further study of the complex remodeling of cellular capabilities when fungi are subjected to limiting nutrient conditions should continue to reveal new regulatory mechanisms. A major unsolved area concerns the identity and/or functional details of the nature of the sensor for sulfur, phosphorus, and iron in fungal systems. In other words, exactly what molecule is being bound by which specific protein acting as a sensor (assuming a direct effect) and how is the signal transmitted to the regulatory network?

Clearly, the expanding availability of genomic information from a diverse set of fungi represents a significant opportunity for the integration of accumulated data from the model fungal systems to examine the nutrient-gathering mechanisms of pathogenic species. Recent studies indicate that new regulatory insights and system-wide dynamics/connections will continue to be revealed in this area of nutrient acquisition.

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26

Secondary Metabolism

B. GILLIAN TURGEON AND KATHRYN E. BUSHLEY

INTRODUCTION

Fungi are physiologically and ecologically diverse organisms, and their ability to produce a vast array of structurally and functionally different small molecules called secondary metabolites is critical to the myriad environmental niches that these organisms occupy. In addition to facilitating survival in various habitats, these metabolites can broker associations with other organisms (other fungi, plants, insects, animals, and humans) and thus can have profound impacts on ecosystem dynamics, world food supply, the economy, human health, etc. Their broad spectrum of known biological activity includes effects that may be detrimental *to other organisms* (e.g., phytotoxins, mycotoxins, carcinogens, etc.) or beneficial *to other organisms* (e.g., antibiotics, inhibitors, immunosuppressants, etc). Until recently, most fungal secondary metabolites were characterized from the perspective of their impact on humans (e.g., they kill our crops, make us sick, save lives, and control pests). Little attention had been paid to the physiological significance of these molecules to the producing fungi (i.e., their natural functions). Contemporary research supports the notion that interorganismal activity is not their only, or even their primary, function and that these metabolites are used by fungi both for basal metabolic activities and for survival/fitness in diverse environmental niches.

Definition and How It Has Changed with Time

In 2008, the Wikipedia entry for secondary metabolites captured the essence of early definitions of these compounds in stating:

> Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms. . . Unlike primary metabolites, absence of secondary metabolites results not in immediate death, but in long-term impairment of the organism's survivability/fecundity. . . Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group.

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We find the first two statements contradictory, since if the second "absence of secondary metabolites results . . . in long-term impairment of the organism's survivability/fecundity" is true, then the first ". . .not directly involved in the normal growth, development or reproduction of organisms" cannot be true. Evidence has accumulated suggesting that secondary metabolite production is intimately connected with development and reproduction, as is demonstrated below.

Although early definitions, built around discoveries of compounds such as the wonder drug penicillin, the crop-destroying, host-selective plant toxins, and the human-health-impacting mycotoxins, were in line with the notion that secondary metabolites are not directly involved in "the normal growth, development or reproduction of organisms," logic tells us that these few "serendipitous" pharmaceutical/crop activities cannot be the natural functions. Fungi do not produce antibiotics with human health issues "in mind." Furthermore, predictions are that there are >1,000,000 different fungal species on the planet (Hawkesworth, 1991); of these, a large percentage are filamentous ascomycetes, and genome mining indicates that these ascomycetes carry ~20 to 40 genes for core biosynthetic enzymes involved in the production of secondary metabolites. Comparative genomics shows that only a few of these genes are conserved across the fungi, which supports the third Wikipedia statement, "Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group." The inescapable conclusion is that the potential for diverse small-molecule production is astounding. Why do fungi carry such a large complement of genes for biosynthesis of this galaxy of different compounds? They do so for development and survival.

Although the term "secondary metabolite" is embedded in the literature, one might argue that it is preferable to use terms such as "natural product" or "small molecule," etc. Whatever the preferred term, the task is to avoid imbuing the word "secondary" with undertones of "unimportant" and rather to think of these compounds from the perspective of growth, development, and survival/fitness of the producing organism.

History of Discovery

The history of identification and practical use of secondary metabolites began with the discovery of penicillin by

Alexander Fleming in 1929. The story of its refinement is not recounted here, as this fascinating history is traced in detail by Demain and Fang (2000), who note,

> "The importance of penicillin was that it was the first successful chemotherapeutic agent produced by a microbe. The tremendous success attained in the battle against disease with this compound not only led to the Nobel Prize being awarded to Fleming, Florey, and Chain, but to a new field of antibiotics research, and a new antibiotics industry. Penicillin opened the way for the development of many other antibiotics, and yet it still remains the most active and one of the least toxic of these compounds.

Following recognition of the antibiotic effects of secondary metabolites was the finding that these compounds have other practical activities, such as acting as inhibitors or immunosuppressants (Omura, 1992; Umezawa, 1972); both findings have advanced treatment of a variety of medical conditions. For example, high cholesterol may be treated with the inhibitor Lovastatin, a polyketide metabolite produced by *Aspergillus terreus*, while the immunosuppressant peptide cyclosporin, produced by *Tolypocladium inflatum*, facilitates organ transplants (Borel, 2002). As noted by Demain and Fang (Demain and Fang, 2000), screening strategies had "one important concept in common, i.e., that microbial metabolites have activities other than, or in addition to, inhibition of other microbes." Note, however, that this comment still refers to useful effects on other organisms.

Shortly after the discovery of penicillin and its beneficial aspects for humans, the detrimental properties of secondary metabolites were revealed upon identification of host-selective toxins (HSTs): e.g., AK-toxin in 1933 (Tanaka, 1933), victorin in 1947 (Meehan and Murphy, 1947), HC-toxin in 1965 (Scheffer and Ullstrup, 1965), T-toxin in 1972 (Yoder and Mukunya, 1972; Yoder, 1973), and mycotoxins such as trichothecenes in 1961 (Brian et al., 1961; Desjardins, 2006). These discoveries ushered in a period of activity centered on elucidation of the physiological properties of the toxins, attempts to understand the genetics of toxin production, and efforts to clone the gene(s) responsible when molecular genetic tools became available. Commencing with the year 2000, the landscape changed dramatically, as genome sequencing revealed that most filamentous ascomycetes carry numerous genes encoding enzymes for secondary metabolite biosynthesis (Kroken et al., 2003; Yoder and Turgeon, 2001). Functional analyses revealed that these molecules not only have effects on other organisms, but as a group are also intimately involved in fungal development and fitness/survival in specialized environmental niches.

Scope of Chapter

In this chapter, several contemporary questions are considered that have arisen as genome sequencing and genomic resources have propelled the field of secondary metabolism to the forefront of fungal biology. The approach is to use case studies to illustrate areas currently under consideration. The chemistry of the compounds, biochemical pathways, and drug discovery are not covered in detail (for examples of this literature, see *Natural Products Reports*, *Nature Chemical Biology*, *Chembiochem*, etc.).

There are four main classes of fungal compounds considered to be secondary metabolites: polyketides (PKs), nonribosomal peptides (NRPs), terpenoids, and alkaloids. The focus in subsequent sections is mainly on PKs and NRPs, as these constitute the two most prominent classes.

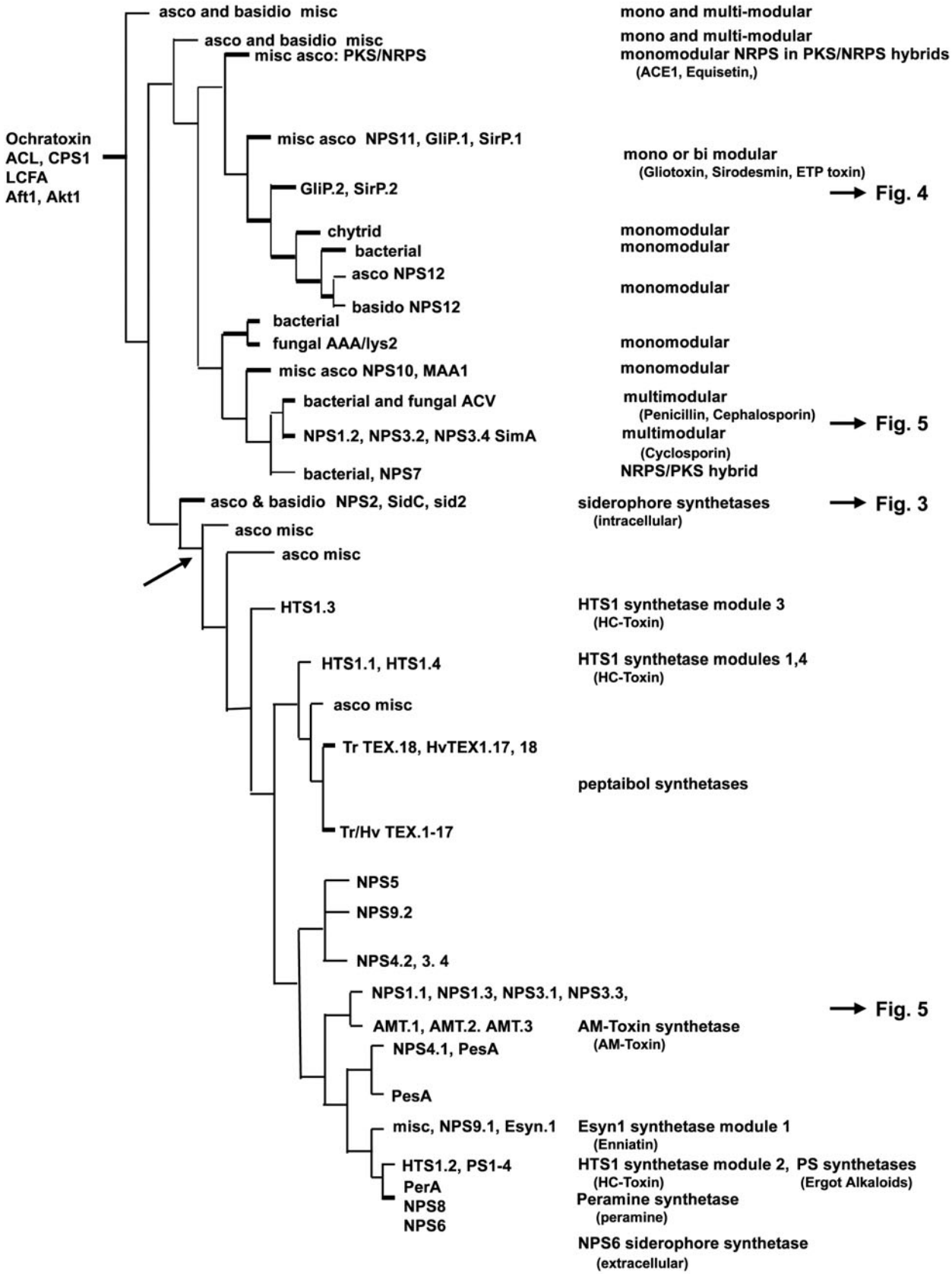
For reviews or primary articles of note on PK synthases (PKSs) and PK biosynthesis, see Chooi et al., 2008; Collemare et al., 2008; Cox, 2007; Gaspar et al., 1999; Hutchinson, 1999; Hutchinson et al., 2000; Kroken et al., 2003; Pankewitz and Hilker, 2008; Stocker-Wörgötter, 2008; Tran et al., 2008; Weissman, 2008; Wolpert et al., 2002; and Zhou et al., 2008. On NRP synthetases (NRPSs) and NRPs, see Caboche et al., 2008; Finking and Marahiel, 2004; Jegorov et al., 2006; Kleinkauf and Von Doehren, 1996; Lautru and Challis, 2004; Marahiel, 1997; Marahiel et al., 1997; Schwarzer et al., 2003; Stack et al., 2007; Turgeon et al., 2008; von Dohren, 2004; and Yonus et al., 2008. On hybrid polyketide/nonribosomal or nonribosomal peptide/polyketide biosynthesis, see Chiang et al., 2008; Dean et al., 2005; Fudal et al., 2007; Kroken et al., 2003; Lee et al., 2005; and Sattely et al., 2008. On terpene/terpenoid biosynthesis, see Saikia et al., 2006, 2008; and Tudzynski, 2005. Finally, on alkaloid biosynthesis, see Fleetwood et al., 2007; Gaspar et al., 1999; Haarmann et al., 2008; Lorenz et al., 2007; Markert et al., 2008; Schardl, 2001, 2006; Schardl et al., 2007; and Tudzynski et al., 1999, 2001.

GENES, ENZYMES, AND SECONDARY METABOLITES

Structure of the Core Enzymes

NRPs

NRPs are peptides synthesized enzymatically by NRPSs, without the use of the ribosomal machinery. In terms of enzyme organization, a minimal NRPS module is composed of an adenylation (A), a thiolation (T, also called peptidyl carrier protein [PCP]), and a condensation (C) domain (Mootz et al., 2002; Schwarzer and Marahiel, 2001). The A domain is required for amino acid substrate recognition and activation as an aminoacyl-*O*-AMP using ATP, while the T domain is the site for phosphopantetheine (PPT) cofactor binding. The holoenzyme then activates aminoacyl substrates to form a thioester bond (Grunewald and Marahiel, 2006). The PPT moiety is added posttranslationally to an invariant serine in a highly conserved region of the T domain (Balibar et al., 2005; Stack et al., 2007). The C domain, typically found after each A-T module, functions in peptide bond formation and elongation of the nascent peptide (Balibar et al., 2005; Marahiel, 1992; Marahiel et al., 1997). The number and order of modules may determine the length and structure of the resulting NRP; however, for fungal NRPSs, more often than not there are fewer complete modules than product constituents, which indicates that some module domains may be used iteratively (Glinski et al., 2002; Mootz et al., 2002). In addition to A, T, and C domains, there may be an *N*-methyltransferase (M) or an epimerase (E) domain that changes an amino acid from L- to D-form. In some NRPSs, a thioesterase (TE) domain is found at the C-terminal end of the protein for release of the NRP from the NRPS and also for enzymatic cyclization of the molecule (Cosmina et al., 1993). Most known fungal NRPS enzymes lack a TE domain. The peptide can be also released by an R domain that reduces the thioester bond to a terminal aldehyde or alcohol (Sims and Schmidt, 2008). The diversity of possible NRP products is potentially limitless, as NRPs can be composed of D- and L-amino acids, protein and nonprotein amino acids, hydroxy acids, ornithine, β-amino acids, and other unusual constituents (Schwarzer et al., 2003).


asco and basidio misc
mono and multi-modular
asco and basidio misc
mono and multi-modular
misc asco: PKS/NRPS
monomodular NRPS in PKS/NRPS hybrids
(ACE1, Equisetin,)
Ochratoxin
ACL, CPS1
LCFA
Aft1, Akt1
misc asco NPS11, GliP.1, SirP.1
mono or bi modular
(Gliotoxin, Sirodesmin, ETP toxin)
GliP.2, SirP.2
Fig. 4
chytrid
monomodular
bacterial
monomodular
asco NPS12
monomodular
basido NPS12
bacterial
fungal AAA/lys2
monomodular
misc asco NPS10, MAA1
monomodular
bacterial and fungal ACV
multimodular
(Penicillin, Cephalosporin)
NPS1.2, NPS3.2, NPS3.4 SimA
Fig. 5
multimodular
(Cyclosporin)
bacterial, NPS7
NRPS/PKS hybrid
asco & basidio NPS2, SidC, sid2
siderophore synthetases
(intracellular)
Fig. 3
asco misc
asco misc
HTS1.3
HTS1 synthetase module 3
(HC-Toxin)
HTS1.1, HTS1.4
HTS1 synthetase modules 1,4
(HC-Toxin)
asco misc
Tr TEX.18, HvTEX1.17, 18
peptaibol synthetases
Tr/Hv TEX.1-17
NPS5
NPS9.2
NPS4.2, 3. 4
NPS1.1, NPS1.3, NPS3.1, NPS3.3,
Fig. 5
AMT.1, AMT.2. AMT.3
AM-Toxin synthetase
(AM-Toxin)
NPS4.1, PesA
PesA
misc, NPS9.1, Esyn.1
Esyn1 synthetase module 1
(Enniatin)
HTS1.2, PS1-4
HTS1 synthetase module 2, PS synthetases
(HC-Toxin)
(Ergot Alkaloids)
PerA
NPS8
Peramine synthetase
(peramine)
NPS6
NPS6 siderophore synthetase
(extracellular)

The structure of each NRPS in each fungus is usually unique (Lee et al., 2005; Turgeon et al., 2008), and both monomodular and multimodular NRPSs are found. Monomodular NRPSs may terminate in domains other than C (e.g., dehydrogenase and NAD-binding domain, Fe reductase transmembrane domain, PKS, etc.) (Lee et al., 2005; K. E. Bushley and B. G. Turgeon, unpublished data).

PKSs

PKs are synthesized enzymatically by PKSs. Fungal PKSs are closely related to fatty acid synthetases (FASs) (Hopwood, 1997; Hopwood and Khosla, 1992; Hutchinson, 1999, 2003). With few exceptions, all fungal PKSs are type I, consisting of a single multidomain enzyme carrying all catalytic domains, rather than the alternative configuration, in which catalytic domains are on separate proteins (type II). Type I PKS domains may be used iteratively (Castoe et al., 2007).

PKSs and FASs are multifunctional enzymes with the same enzymatic domain structure. The ketoacyl synthase, acyltransferase, and acyl carrier protein domain (also known as a PPT attachment site [PP] domain) are essential for both FASs and PKSs, whereas the ketoreductase, dehydratase, and enoyl reductase domains are present in all FASs, but some or all are absent in various PKSs. The ketoreductase, dehydratase, and enoyl reductase domains catalyze, in a stepwise fashion, reduction of a keto to a hydroxyl group, dehydration of the hydroxyl to an enoyl group, and reduction of the enoyl to an alkyl group, respectively. PKSs that lack some or all of these domains produce partially reduced or fully oxidized PKs. Thus, the diversity of PKs is generated, in part, through the use of the three optional PKS reducing domains. Fungal type I PKSs, like FASs, are monomodular enzymes; most are iterative and use their active sites repeatedly to synthesize a PK, adding a two-carbon molecule (i.e., a coenzyme A [CoA] ester) to the growing chain with each condensation.

Terpene Synthases

All terpenes are polymers of repeating isopentyl units built by prenyltransferases. Monoterpenes are derived from geranyl diphosphate (GPP), sesquiterpenes are derived from farnesyl diphosphate, and diterpenes are derived from geranylgeranyldiphosphate (GGPP) by the action of terpene synthases or cyclases (Toyomasu, 2008). Indole-diterpenes have a cyclic diterpene backbone derived from GGPP and an indole group derived from indole-3-glycerol phosphate (Saikia et al., 2008). As noted by Parker and Scott (Parker and Scott, 2004), the "presence of two copies of GGPP synthases in a genome is thought to be a 'signature' for fungi capable of producing diterpenes, one for primary and one for secondary metabolism" (Parker and Scott, 2004; Saikia et al., 2008). Indole-diterpenes include those having mammalian neurotoxic effects and those providing an ecological advantage to fungal symbionts. Examples include the *Gibberella fujikuroi* diterpene product, gibberellic acid (Gaspar et al., 1999; Young et al., 2001, 2005; Tudzynski and Holter, 1998), which affects plant growth, the *Fusarium graminearum* sesquiterpene mycotoxins (Hohn and VanMiddlesworth, 1986), and the *Penicillium paxilli* metabolite, paxilline (Saikia et al., 2007), which can block Ca^{2+}-activated K^+ channels.

Alkaloid Synthases

Alkaloids are secondary metabolites containing one or more basic nitrogen atoms bound to carbon as well as hydrogen, generally in a heterocyclic ring (da Silva et al., 2007; Michael, 2008). The chemical definition is imprecise, making it difficult to distinguish them from other types of organic nitrogen-containing compounds. The best known are those produced by fungal grass endophytes such as the ergot fungus, *Claviceps purpurea* (Lorenz et al., 2007; Schardl, 2001; Schardl et al., 2007). Ergot alkaloid toxins are assembled from prenylated tryptophan and include clavines, lysergic acid, and derivatives thereof. NRPSs are often involved.

Evolutionary Relationships

As a basis for further discussion, we have included diagrams of phylogenetic trees built with the A domains of NRPSs (Bushley and Turgeon, unpublished) (Fig. 1) and the ketoacyl synthase domains of PKSs (redrawn from Kroken et al., 2003) (Fig. 2). At the time of the earlier publication, there were only a few complete genome sequences available; however, results from these analyses remain unchanged, despite the larger number of genomes now available. Where possible, fungi representing all major taxonomic groups and lifestyles (pathogens/saprobes, plant/animal pathogens, etc.) were included in tree building.

These two tree skeletons are the foundation for the rest of this article. From the original trees, the following points are made. (i) The central genes, *NPS* and *PKS*, are highly diverse and rapidly evolving. Few are conserved across the fungi. (ii) Most group with other fungal, not with bacterial, genes. (iii) Many are in gene clusters. (iv) Where they have been examined closely, these genes tend to reside near the ends of supercontigs/chromosomes, or in high single nucleotide polymorphism (SNP) or recombination regions. (v) Some NRPS proteins are monomodular, others are multimodular. Monomodular NRPSs are likely ancestral. For multimodular NRPSs, the A domains of any given protein may or may not be each other's closest relative. (vi) Multimodular NRPSs and multidomain PKSs are found in abundance in genomes of Ascomycetes, suggesting niche-related amplification in this group. (vii) The domain structure of each NRPS protein is usually unique in a given genome, unlike PKSs. (viii) There are two major groups of PKSs corresponding to reducing or nonreducing activities: within each of these are a variety of protein structures. Both Fig. 1 and Fig. 2 refer to additional figures which are used to address topics in the sections below.

Conserved versus Discontinuous Distribution

The level of conservation of members of each type of NRPS or PKS varies widely; however, it is clear that while a few

FIGURE 1 Cartoon of maximum likelihood phylogenetic tree built with individual A domains of NRPSs extracted from fungal genomes for which genome sequences are available (Bushley and Turgeon, unpublished [available on request]). Also included were A domains from selected NRPSs deposited in GenBank. All *C. heterostrophus* NRPSs are included (designated NPS1-12) (Lee et al., 2005). Thick bars indicate robust support; the arrow indicates that NRPSs from all taxa below are from filamentous ascomycetes. Note that mono-or bimodular NRPSs dominate the top of the tree, while multimodular NRPSs populate the bottom. Note that Fig. 3 through 5 are cross-referenced.

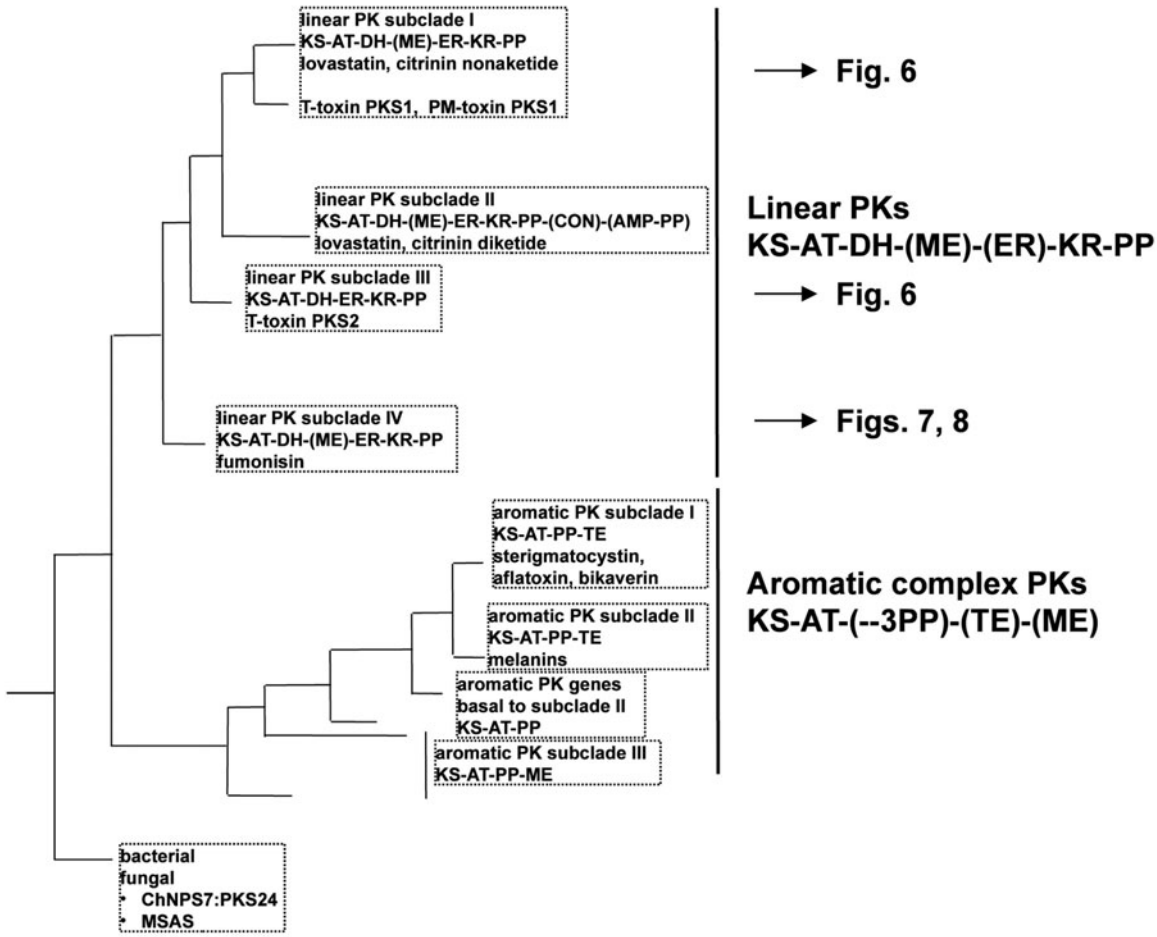


FIGURE 2 Cartoon of phylogenetic tree built with individual ketoacyl synthase domains of PKSs as reported by Kroken et al. (2003). Note that Fig. 6 through 8 are cross-referenced.

are conserved, most show a highly discontinuous distribution across filamentous fungal genomes. Yeasts have few NRPSs or PKSs, if any (Lee et al., 2005). Among 12 *NPS* genes in the filamentous ascomycete *Cochliobolus heterostrophus*, only four are conserved (all are shown in Fig. 1 and in the work of Lee et al. [2005]). These include multimodular NPS2 (Fig. 3A) (Oide et al., 2007), which is responsible for intracellular siderophore production and whose absence affects sexual reproduction. This NRPS is the most conserved of all and is found in both ascomycetes and basidiomycetes (Fig. 1 and 3A). Bimodular NPS6 (Fig. 1 and 3B (Oide et al., 2006), responsible for extracellular siderophore production, virulence, and resistance to oxidative stress and to low-iron conditions, is conserved in most ascomycetes examined to date, but not in basidiomycetes. Multimodular NPS4 is widely present in ascomycetes but not basidiomycetes and has been shown to control cell surface hydrophobicity in *C. heterostrophus*, *Alternaria brassicicola*, and *Gibberella zeae/Fusarium graminearum* (Fig. 1) (Kim et al., 2007; Turgeon et al., 2008) and conidial properties in *A. brassicicola* (Kim et al., 2007). Monomodular NPS10 (Fig. 1) is present in many ascomycetes and a few basidiomycetes. In *C. heterostrophus*, *A. brassicicola*, and *G. zeae*, its absence affects colony morphology and resistance to oxidative stress (Oide, 2007; Turgeon et al., 2008). Significantly, the metabolites produced by conserved NPS2, NPS6, NPS4, and NPS10 affect fundamental characteristics of fungal cells or critical aspects of metabolism (e.g., resisting stress, nutrient gathering, and reproduction [Turgeon et al., 2008]).

The *C. heterostrophus NPS11* gene encodes a monomodular NRPS, which has an intermediate level of conservation (Fig. 1). Homologs are found in several ascomycetes and include the *Aspergillus fumigatus* (human pathogen) genes encoding bimodular NRPSs for gliotoxin (with a possible role in virulence [Cramer et al., 2006; Spikes et al., 2008; Sugui et al., 2007]) and ETP toxin production, the *Leptosphaeria maculans* (plant pathogen) gene encoding a bimodular NRPS for sirodesmin production, and the bimodular (nonpathogenic) *Trichoderma reesei* genes encoding NRPSs that appear to be homologs of the *A. fumigatus* genes encoding NRPSs for gliotoxin and ETP toxin (Fig. 4). *C. heterostrophus* NPS11 and its homologs in *Magnaporthe oryzae* and *Botrytis cinerea* (plant pathogens) are monomodular, and these single modules group with module 1 of bimodular GliP, SirP, and the *T. reseei* protein (24586). The second module of Glip, SirP, and *T. reesei* 24586 groups closely with monomodular NRPSs that occur in ascomycetes, basidiomycetes, chytrids, and bacteria, suggesting deep ancestry (Fig. 1 and Fig. 4). This grouping also includes *C. heterostrophus* NPS12 (Fig. 1).

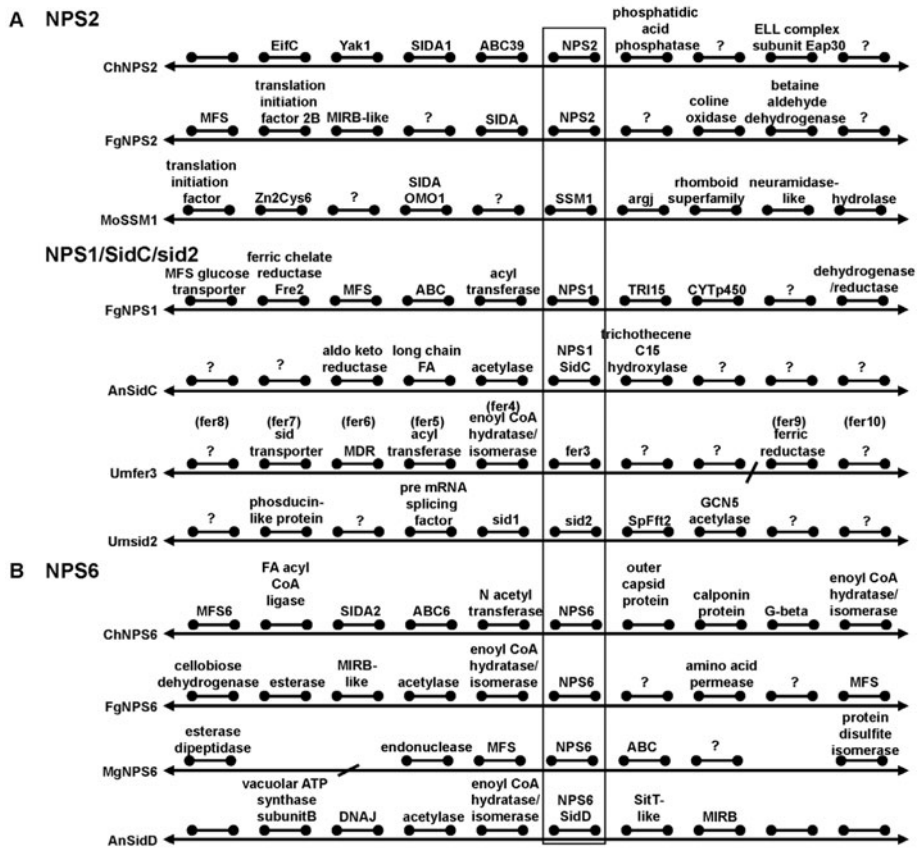


FIGURE 3 Comparison of neighborhoods surrounding the conserved NRPSs for intracellular (NPS2/NPS1/SidC/sid2 [A]) and extracellular (NPS6 [B]) siderophore biosynthesis. Gene annotations for *C. heterostrophus* are from JGI (http://genome.jgi-psf.org/CocheC5_1/CocheC5_1.home.html), while the rest are from BROAD (http://www.broad.mit.edu/node/568). Note that for both NRPS lineages, which are the most conserved in filamentous fungi, some, but not all, pathway genes are present (as described in the text); otherwise, flanking genes are not conserved. The siderophore-producing NPS2/NPS1/SidC/sid2 NRPSs are found in both ascomycetes and basidiomycetes, and there is good support for this clade grouping with the multimodular NRPSs in clades found only in filamentous ascomycetes (Fig. 1).

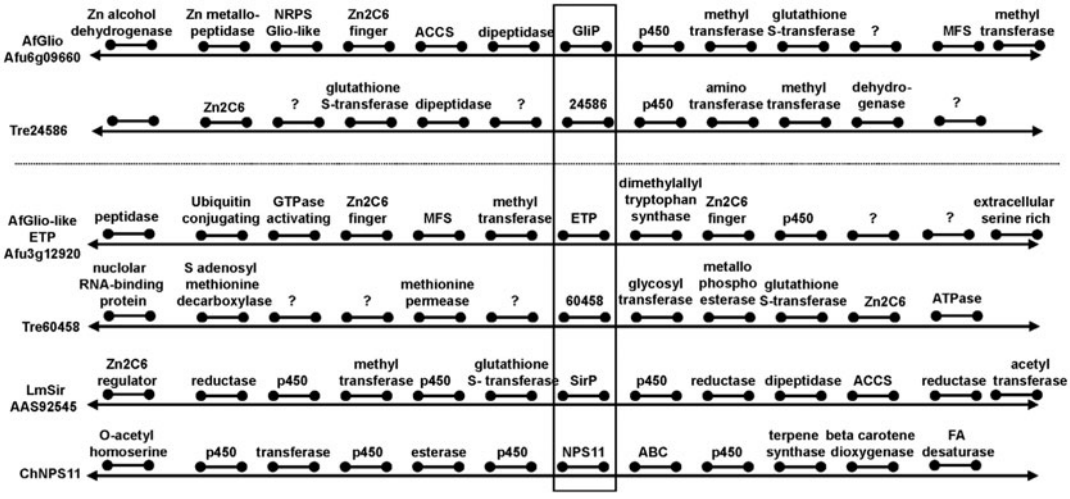


FIGURE 4 Comparison of neighborhoods surrounding the moderately conserved mono- and bimodular NRPSs (Fig. 1), found in only a few filamentous ascomycetes. Monomodular NPS11 from *C. heterostrophus* groups with module 1 of the NRPSs for ETP and sirodesmin, while monomodular NPS12 from *C. heterostrophus* groups with module 2 of these NRPSs and with monomodular NRPSs from bacteria, chytrids, ascomycetes, and basidiomycetes (Fig. 1). While the predicted proteins of genes adjacent to *ChNPS11* are typical of those involved in secondary metabolism, they are different from those at the ETP/sirodesmin loci (where several genes are conserved across all).

The highly discontinous distribution of the central genes (e.g., *PKS*s and *NPS*s) encoding enzymes for biosynthesis of secondary metabolites means that metabolites with closely related chemistry may be produced by highly divergent taxa with little or no evidence for these compounds (or the corresponding genes) in intervening taxa. We use several examples to illustrate this point. At the far end of the spectrum are the multimodular NRPSs for host-selective toxins such as *Cochliobolus carbonum* HC-toxin and *Alternaria alternata* AM-toxin, which are found in specific races/pathovars of a single species only (Johnson et al., 2000; Scottcraig et al., 1992) (Fig. 1). Without these genes, the corresponding fungus is nonpathogenic; thus, the product is critical to pathogen development on the host. To date, there is no A domain from any other known NRPS that is a close match to any of the A modules of the tetramodular NRPS HST1 for HC-toxin production. For the A domains of trimodular AMT synthetase, the phylogeny reveals that two A domains of *C. heterostrophus* NPS1 and two of NPS3 are closely related (Fig. 1 and 5A). *C. heterostrophus NPS1* and *NPS3* encode tri-and tetramodular NRPSs, respectively. There are no complete NPS1 or NPS3 counterparts in closely related *Cochliobolus* species. NPS1 modules 1 and 3 and NPS3 modules 1 and 3 group with the three A modules of AMT (Fig. 5A), while NPS1 module 2 and NPS3 modules 2 and 4 group separately and are within the SimA (encoding cyclosporin) clade (Fig. 5B). There is no known function or suggested metabolite for NPS1 or NPS3; however, AMT toxin is required for pathogenicity of *A. alternata* on its host, apple, and cyclosporin acts as an immunosuppressant. No function for the benefit of the producing fungus is known. There is evidence that cyclosporin can bind calcium, zinc, and copper, suggesting perhaps that it acts as an ionophore (Cusack et al., 2003; Dancer et al., 1995). ChNPS1 and ChNPS3 have both been deleted in *Cochliobolus*, and mutants have been screened for a variety of phenotypes (including a role in virulence), but none has been found. Recent data indicate that *ChNPS1* expression is elevated under low-Zn conditions (N. Zhang and B. G. Turgeon, unpublished data).

The SIMA cyclosporin clade reveals an additional point. It is clear that such an expanded clade (Fig. 1 and 5B), with 11 A domains and as many modules, groups with mono and bimodular A domains from other fungal NRPSs. We suggest that the monomodular associates are the ancestral configuration and that multimodular SIMA arose by repeated module duplication.

For PKSs, distribution is spotty. Of the 25 *C. heterostrophus PKS*s, astonishingly, none is conserved across all major phylogenetic groupings (see Fig. 1 in Kroken et al., 2003), except for the *PKS* in the melanin pathway. For example, two of the *C. heterostrophus PKS*s (*ChPKS1* and *ChPKS2*) (Fig. 6A) are unique to race T, one of two races of *C. heterostrophus* distinguished by the ability to make (race T) or not make (race O) T-toxin. Both *ChPKS1* and *ChPKS2* are required for T-toxin production. Neither is in race O, and both are missing from all other known *Cochliobolus* and closely related species. *ChPKS1* and *ChPKS2* are not closely related to each other (Fig. 2

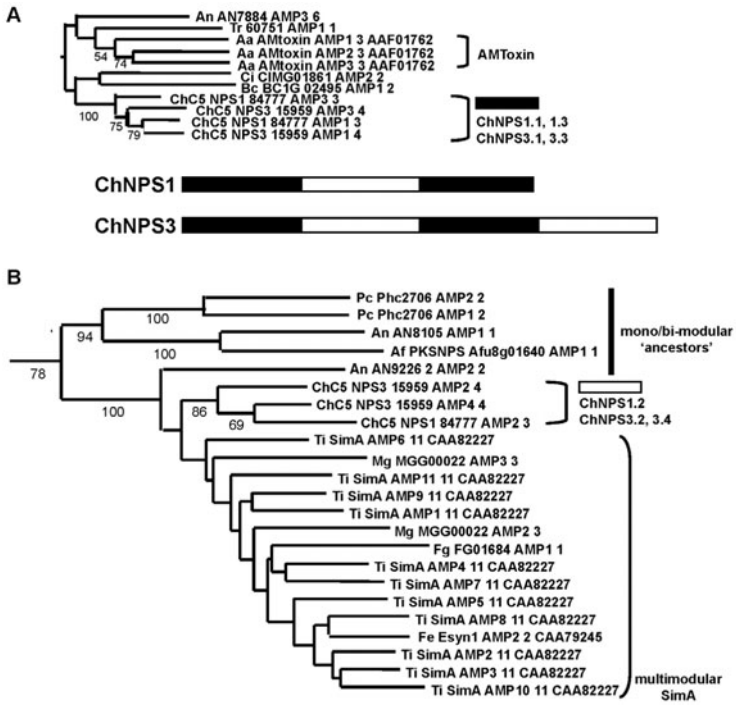


FIGURE 5 Discontinuously distributed, multimodular ChNPS1 and ChNPS3 are related, and some modules of each enzyme group in the SIMA clade (B) for cyclosporin biosynthesis, while others group in an unrelated clade (A) (Fig. 1). The monomodular members related to SIMA suggest that the multimodular SIMA NRPS arose by repeated duplication. NPS1 and NPS3 evolution must have been more complicated and involved duplication and recombination. Neither gene has a complete counterpart in any other known genome sequence.

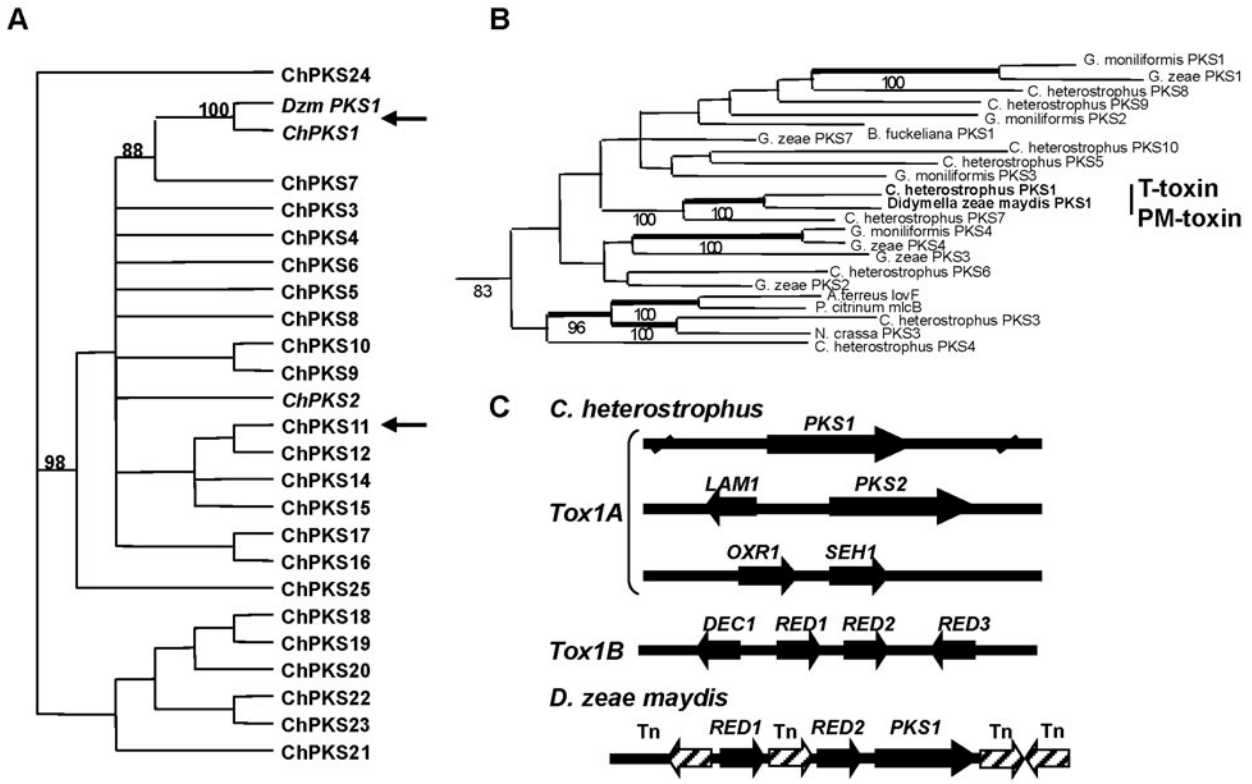


FIGURE 6 The PKSs involved in HST production are unique. (A and B) *C. heterostrophus* race-T genome sequence reveals 25 *PKSs*. *PKS1* and *PKS2* are unrelated, but both are required for T-toxin production. Neither occurs in race O. The closest known PKS is *PKS1* of *D. zeae-maydis*. (C) Comparison of the loci for T-toxin production in *C. heterostrophus* and for PM-toxin production in *D. zeae-maydis*. The *Tox1* locus of *C. heterostrophus* is two loci (*Tox1A* and *Tox1B*) on two different chromosomes, while the *Tox* locus of *D. zeae-maydis* is single. Only the *PKS1* genes are orthologous. For gene designations see Turgeon and Baker (2007).

and 6A). The closest match to *ChPKS1* is *PKS1* of *Didymella zeae-maydis* (*Mycosphaerella zeae-maydis*, *Phyllosticta maydis*) (Fig. 6B), an unrelated Dothideomycete that makes PM toxin, a family of PKs with the same biological activity and specificity as T-toxin. *D. zeae-maydis* does not appear to have a PKS2 counterpart (Turgeon and Baker, 2007). Furthermore, the genome sequence neighborhood surrounding the two orthologous *PKSs* (*ChPKS1* and *DzmPKS1*) is not conserved (Fig. 6C). Before the *C. heterostrophus* genome sequence became available, horizontal transfer was invoked to explain the irregular distribution (Rose et al., 2002; Yang et al., 1996). However, the *Cochliobolus PKS1* and *PKS2* genes have the same structural characteristics as other *PKS* genes in *Cochliobolus* and other fungi, all or most group with other fungal genes, and all show sporadic distribution. Thus, there is no compelling evidence for horizontal transfer of these genes (Kroken et al., 2003). Mechanisms contributing to the discontinuous distribution are discussed below.

A few discontinuously distributed *PKSs* are like *ChPKS11*. The ortholog in *G. fujikuroi/Fusarium verticillioides*, *FUM1*, encodes a PKS in a 15-gene cluster responsible for fumonison production (Fig. 7). *C. heterostrophus* not only harbors an ortholog of *FUM1* (*ChPKS11*) but also has 10 of

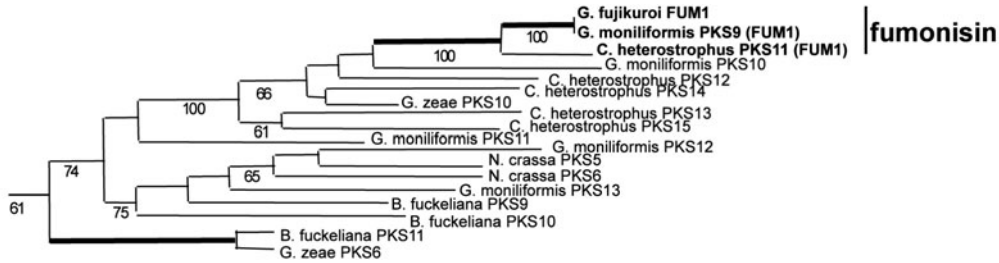


FIGURE 7 *C. heterostrophus PKS11* is an ortholog of *G. fujikuroi/F. verticillioides FUM1*.

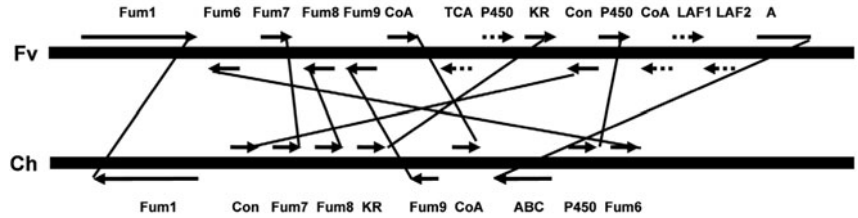


FIGURE 8 Ten of the 15 genes at the *G. fujikuroi/F. verticillioides FUM1* locus are conserved in *C. heterostrophus*.

the 15 *FUM1* cluster genes, although gene order is not conserved (Fig. 8). A *FUM1/ChPKS11* ortholog is also present in *A. alternata* and is responsible for AAL toxin production (M. Kodama, personal communication). The observation that both fumonison and AAL toxin inhibit ceramide synthesis raises the question as to whether the ChPKS11-associated metabolite has similar activity.

In summary, the distribution of *PKS* and *NPS* genes varies widely within the fungi. Most yeasts lack these genes, and most basidiomycetes have only a few, while most filamentous ascomycetes have many. Most of the genes are not conserved. Where homologs are found in unrelated fungi, percent similarity is low, indicating the genes are fast evolving. The neighborhood around the homologous core *PKS* or *NPS* is often shuffled and/or unrecognizable.

Are Core PKS and NPS Genes for Secondary Metabolism Often in Clusters?

The identification of genes encoding core PKSs and NRPSs for production of secondary metabolites has revealed that they may be clustered with other genes that are part of the same metabolic pathway and that gene expression in such clusters is coregulated (Keller and Hohn, 1997). One of the first examples was the *Aspergillus nidulans* 60-kb region carrying the 25 genes required for sterigmatocystin biosynthesis (Brown et al., 1996); all 25 are coexpressed under inducing conditions, while genes flanking this cluster are expressed under both inducing and noninducing conditions. Interestingly, with respect to both the cluster issue and the discontinuous distribution issue, the PK toxin dothistromin, produced by *Dothistroma septosporum*, is structurally similar to the aflatoxin precursor, versicolorin B. Orthologs of aflatoxin biosynthetic genes, required for dothistromin biosynthesis, occur in three miniclusters rather than one (Zhang et al., 2007). Most are coregulated, suggesting that control of gene expression is achieved despite the tripartite arrangement.

As noted above, genome sequencing has revealed that core *PKS*s and *NPS*s associated with secondary metabolism are plentiful in filamentous ascomycete genomes; for most, however, the corresponding metabolites are unknown. With respect to the issue of whether or not all are situated in coregulated gene clusters, except for the *Aspergillus* examples and a handful of others (for an example, see Kutil et al., 2007), most have not been experimentally verified. How

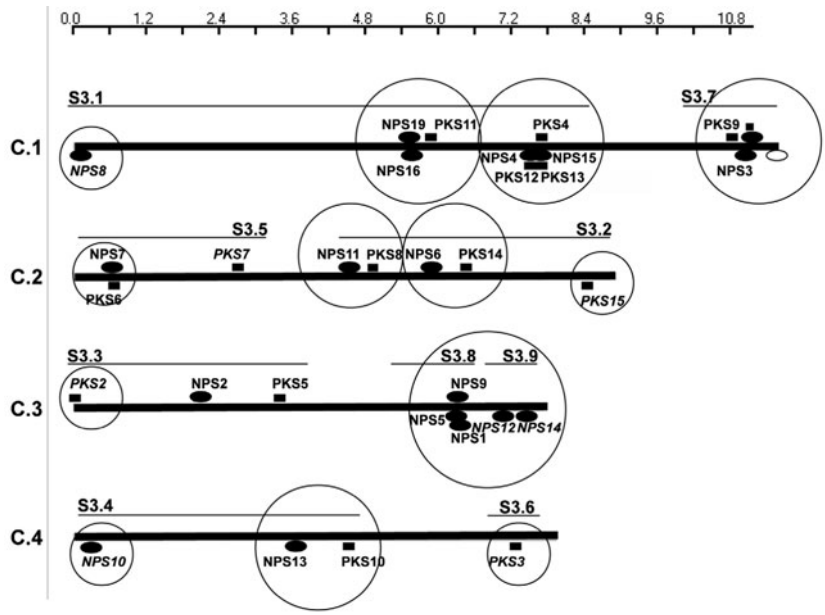


FIGURE 9 *G. zeae PKS*s and *NRPS*s tend to map to supercontig ends. Shown are the four *G. zeae* chromosomes (C.1 through C.4) and *PKS* (squares) or *NPS* (ovals) gene locations, adapted from Cuomo et. al. (2007). Lines above the chromosomes represent supercontigs; "S" indicates supercontig number, and placement indicates first nucleotide of the sequence. Circles indicate high SNP or recombination regions. Scale is in megabases.

does one delineate a cluster? If the product is known, a gene knockout approach could be taken with each candidate gene in a cluster to determine if metabolite production is altered. In the absence of knowledge of the metabolite product, conditions might be found under which an identified *PKS* or *NPS* is expressed, and then the rest of the genes in a proposed cluster can be examined for similar expression patterns. Alternatively, if there is a pathway-specific regulator, such as the $Zn(II)_2Cys_6$ DNA binding domain transcription factor AflR, in the sterigmatocystin/aflatoxin pathway (Chang et al., 1995; Fernandes et al., 1998; Gaspar et al., 1999; Woloshuk et al., 1994; Yu et al., 1996), it could be deleted and expression effects on the neighborhood genes could be examined. This approach was taken to identify the borders of the fumonisin (FUM1) (Fig. 8) biosynthetic gene cluster of *F. verticillioides* (Brown et al., 2007), the cercosporin biosynthetic cluster in *Cercospora nicotianae* (Chen et al., 2007), and the aurofusarin biosynthetic gene cluster (Fig. 9 and Table 1) in *G. zeae* (Kim et al., 2006).

A significant advance in cluster demarcation was made a few years ago when a global regulator of secondary metabolism (LaeA) (see "Regulation" below) was identified in *A. nidulans* (Bok and Keller, 2004). Deletion of this gene alters (eliminates or reduces) expression of many, but not all, proposed gene clusters in *A. nidulans* and *A. fumigatus*. Thus, comparison of wild-type and *lae*A mutant expression profiles can pinpoint cluster signatures efficiently across the genome, assuming genomic resources are available. As far as we are aware, LaeA has not been shown to be involved in secondary metabolite regulation in additional taxonomic groups. Another approach to altering production of a number of secondary metabolites at once is to manipulate the 4′-phosphopantetheinyl transferase (PPTase) gene, which makes a cofactor required

TABLE 1 *F. graminearum* PKS and NPS locations

Gene[a]	Genome ID	Coordinates	Distance (kb) from supercontig end	Supercontig	Chromosome
NPS1	FGSG_11026	574013–588321		3.8	3
NPS2	FGSG_05372	2043338–2057970		3.3	3
NPS3	FGSG_10523	2145297–2137744		3.7	1
NPS4	FGSG_02315	7462127–7439208		3.1	1
NPS5	FGSG_13878	693139–727216		3.8	3
NPS6	FGSG_03747	2815749–2809478		3.2	2
NPS7	FGSG_08209	2582878–2569253		3.5	2
NPS8	FGSG_11659	158252–144805	~160	3.1	1
NPS9	FGSG_10990	689261–686748		3.8	3
NPS10	FGSG_06507	269866–266018	~250	3.4	4
NPS11	FGSG_03245	4166150–4162913	~1,000	3.2	2
NPS12	FGSG_11294	577120–580295	~170	3.9	3
NPS13	FGSG_13153	3631142–3627883		3.4	4
NPS14	FGSG_11395	312651–319899	~300	3.9	3
NPS15	FGSG_02394	7677408–7670431		3.1	1
NPS16	FGSG_01680	5539722–5534719		3.1	1
NPS17	FGSG_10702	2682687–2680651	~500	3.7	1
NPS18	FGSG_13783	2214527–2239324	~500	3.7	1
NPS19	FGSG_11989	5556950–5560682		3.1	1
PKS1	FGSG_10548	2251930–2259838	~500	3.7	1
PKS2	FGSG_04694	17501–25708	~20	3.3	3
PKS3	FGSG_09182	726281–719614	~700	3.6	4
PKS4	FGSG_12126	7689276–7696151		3.1	1
PKS5	FGSG_05794	3414831–3404771		3.3	3
PKS6	FGSG_08208	2584081–2591852		3.5	2
PKS7	FGSG_08795	588238–580813	~600	3.5	2
PKS8	FGSG_03340	3909452–3900928		3.2	2
PKS9	FGSG_10464	1929075–1937298		3.7	1
PKS10	FGSG_07798	4515787–4503929		3.4	4
PKS11	FGSG_01790	5848155–5855750		3.1	1
PKS12	FGSG_02324	7493164–7486702		3.1	1
PKS13	FGSG_02395	7687743–7681422		3.1	1
PKS14	FGSG_03964	2262979–2256591		3.2	2
PKS15	FGSG_04588	285263–291852	~300	3.2	2

[a]Genes in italics are within 300 kb of a supercontig end.

for both PKS and NRPS biosynthesis (Marquez-Fernandez et al., 2007).

Figure 3A shows a comparison of the neighborhood surrounding the most conserved *NPS* gene in fungi, which corresponds to C. *heterostrophus* NPS2 and its orthologs (Oide, 2007). *NPS2* is found in all filamentous ascomycetes examined to date, in some basidiomycetes, and even in *Schizosaccharomyces pombe*, although most yeasts lack *NPS* genes. Detailed phylogenetic analyses of the proteins (Bushley et al., 2008) have shown that there are two extant NPS2 lineages, one including C. *heterostrophus*, F. *graminearum* NPS2, and M. *oryzae* SSM1 and the second including F. *graminearum* NPS1, A. *nidulans* SidC, and *Ustilago maydis* fer3 and sid2. All are responsible for biosynthesis of ferrichrome siderophores (Haas et al., 2008). The gene neighborhood of the conserved NPS is of particular interest. A gene encoding L-ornithine monooxygenase, the first committed step in the siderophore pathway, is present in the NPS2 lineage (FgsidA, ChsidA1, and MoOMO1) but for the NPS1/SidC/sid2 lineage, only *U. maydis* has a linked version of this gene (*sid1*). *U. maydis*, in fact, has two ferrichrome-producing NRPSs (sid2 for ferrichrome and fer3 for ferrichrome A), and the work of Bushley et al. (2008) indicates that a further duplication in the NPS1/SidC/sid2 lineage accounts for this. Umfer3 is most closely related to FgNPS1 and AnSidC, while Umsid2 is on a separate branch in the same lineage (Bushley et al., 2008). It has been demonstrated that Um *fer3-fer10* genes are coregulated and that *sid1* and *sid2* are coregulated (Eichhorn et al., 2006), but the remainder of the adjacent genes are not (Fig. 3A). This type of expression profiling has been done also for *A. fumigatus* (Schrettl et al., 2008). Under iron-regulated conditions, SidC and an adjacent long-chain fatty acid CoA ligase are coregulated (Fig. 3A). As far as we are aware, expression profiling has not been done for the other ferrichrome-producing clusters.

The conclusion from comparison of the most conserved NRPS in fungi is that in many but not all cases the expected pathway genes (sidA, transporters, acetylases, and acylases) are in the vicinity and, where it has been examined, coexpressed.

The second gene cluster case study is that corresponding to C. *heterostrophus* NPS6 (Fig. 1 and 3B), which is conserved in most filamentous ascomycetes examined to date (Lee et al., 2005; Oide et al., 2006) and is responsible for extracellular siderophore production in the form of coprogens, or TAFC-like metabolites. For C. *heterostrophus* and F. *graminearum*, functional analyses indicate that these metabolites are required for virulence and resistance to oxidative stress and low-iron conditions (Oide et al., 2006). The counterpart in *A. fumigatus* is also required for virulence in mice (Schrettl et al., 2007). NPS6 from the saprobe *Neurospora crassa* can complement a C. *heterostrophus nps6* deletion strain and restore wild-type virulence to maize and resistance to oxidative stress (Oide et al., 2006). In contrast, however, M. *oryzae* SSM1 (NPS2) has been shown to be required for virulence, but the NRPS has not been shown to be required for extracellular siderophore biosynthesis (Hof et al., 2007).

Note that the neighborhood around *NPS6* homologs is variable, although most genomes carry transporters (ABC and MFS), enoyl-CoA isomerases, and acetylases involved in the pathway. Only C. *heterostrophus* and other Dothideomycetes have a second SidA (L-ornithine monooxygenase) gene (*SIDA2*) (S. Oide and B. G. Turgeon, unpublished data). It has been demonstrated that siderophore biosynthesis in *Aspergillus* is controlled by iron status, mediated by the GATA factor SreA. If iron is sufficient, absence of SreA partially derepresses biosynthesis of extracellular TAFC by SidD and uptake of iron, resulting in increased cellular accumulation of both iron and ferricrocin. A recent *A. fumigatus* genome-wide profiling experiment comparing *sreA* and wild-type strains indicated that 49 genes are repressed by iron in an SreA-dependent manner, including all known siderophore biosynthetic genes (both extracellular and intracellular). Thus far, expression profiling of the *NPS6* cluster has not been performed for other fungi. Note also that NPS6 groups closely with NPS8 (no known metabolite product) and with the *Epichloe festucae* PerA NRPS (peramine) (Fig. 1).

The third case study of clusters is of NRPSs in the clade for *A. fumigatus* gliotoxin and *L. maculans* sirodesmin production. These NRPSs group with NRPSs that are mostly mono- or bimodular enzymes (Fig. 1 and 4). *A. fumigatus* and *T. reesei* each have two clusters of this type. Grouping with these are monomodular homologs (e.g., *ChNPS11*) of one or the other of the bimodular GliP and SirP modules. These monomodular homologs are found in only a few of the filamentous ascomycetes for which genome sequences are available, unlike the siderophore- related clusters, described above, that are ubiquitous in filamentous ascomycetes. Expression comparisons have shown that the clusters in *L. maculans* and *A. fumigatus* encode $Zn(II)_2Cys_6$ proteins (GliZ and SirZ) and that disruption of *GliZ* in *A. fumigatus* results in a mutant with significantly reduced transcription of the gliotoxin NRPS that is unable to produce gliotoxin (Bok et al., 2006a). Similarly, RNA-mediated silencing produced *L. maculans* mutants that were very reduced in sirodesmin production and in transcription of several cluster genes (Fox et al., 2008). Although *ChNPS11* groups with the *A. fumigatus*, *T. reesii*, and *L. maculans* genes and the gene is surrounded by others often associated with secondary metabolism (e.g., p450s and ABC), these do not appear to be the same as the genes in the gliotoxin or sirodesmin clusters.

The evolutionary origins and maintenance of gene clusters such as those described above are clearly difficult to unravel and may involve more than one mechanism. Although *Saccharomyces cerevisiae* and other yeasts appear to lack genes for secondary metabolism (Kroken et al., 2003; Lee et al., 2005), a study of the *S. cerevisiae* pathway for allantoin degradation is informative, as it demonstrated that six of eight genes were assembled recently from dispersed genomic locations (Wong and Wolfe, 2005). Formation of the *S. cerevisiae* biotin cluster presents an even more complex scenario involving both gene duplication and horizontal transfer of individual genes, but not the complete pathway, from bacteria (Hall and Dietrich, 2007).

Gene duplication and recruitment of one of the two duplicated genes accompanied by altered function provide a reasonable explanation for the origins of genes in secondary metabolite clusters. Formation of the gene cluster for aflatoxin suggests that several genes in the cluster were recruited from duplicates elsewhere in the genome (Cary and Ehrlich, 2006). The study of ETP clusters (Fig. 4) indicates that cluster genes share closest relationships with paralogous genes elsewhere in the genomes (Patron et al., 2007). Indeed, it has been demonstrated for ETP-producing gene clusters that most cluster genes form monophyletic clades and that noncluster paralogs are the nearest outgroups (Patron et al., 2007). This is also true of at least some of the

genes (e.g., LAF) in the *FUM1* cluster (Fig. 8) (S. Kroken, S. E. Baker, and B. G. Turgeon, unpublished data).

The conclusions are that, where they have been examined, core *NPS* and *PKS* genes are usually in coexpressed clusters and that the evolutionary origins of these clusters are largely unresolved.

Are Core Genes for Secondary Metabolite Biosynthesis at Telomeres?

A popular hypothesis is that secondary metabolite genes and their clusters are located in subtelomeric regions, along with other genetic elements that are fast evolving (e.g., organism-specific genes, including virulence factors), transposons, and repetitive DNA (Cuomo et al., 2007; Dean et al., 2005; Nierman et al., 2005; Wood et al., 2002). It is thought that this location facilitates mechanisms that contribute to the rapid evolution, rearrangement, and/or loss of genes, clusters, or parts of clusters. While data on mapping of telomeres are not available for all genomes and clearly some *NPS*s and *PKS*s do not fall within subtelomeric regions (Nierman et al., 2005) (Fig. 9 and Table 1), a convincing case can be made that there is a subtelomeric bias. A recent study involving *A. fumigatus* identified large subtelomeric genomic islands that contain the majority of lineage-specific genes, including some involved in secondary metabolism (Fedorova et al., 2008). Genome-wide investigation of *F. graminearum* indicates the same pattern (Cuomo et al., 2007). As a case study, we have reexamined the four *F. graminearum* chromosomes and the coordinates of all of the *NPS* and *PKS* genes (Table 1 and Fig. 9). Although it is clear that there is a bias for subtelomeric regions, as documented by Cuomo et al., 2007, if one searches the scaffolds for genes that fall within 1 to 300 kb (as was done by Fedorova et al., 2008) of the end of a supercontig which might coincide with subtelomeric regions, then 6 of the 34 total *NPS* and *PKS* genes fall in that range (*PKS2*, *PKS15*, *NPS8*, *NPS10*, *NPS12*, and *NPS14*). If the window is enlarged to 500 kb, 9 of 34 genes fall in the range (additional genes include *PKS1*, *NPS17*, and *NPS18*). When the program TERMINUS (Li et al., 2005) was used to identify *F. graminearum* telomeres (M. Farman, personal communication), only one of these, *PKS2*, which is 17 kb from the end of supercontig 3.3, was confirmed (Table 1). An added complication attends this analysis, which is the chromosome-internal high-SNP and recombination regions, hypothesized to be *F. graminearum* chromosome fusion junctions. The coordinates of these are not precise; however, the mapping does show a density of *NPS* and *PKS* in these regions (Fig. 9 and Table 1).

Is there a bias for a type of secondary metabolite gene to be found in subtelomeric locations? This is an impossible question to address at present since so few of the 34 products are known. For those genes that are clearly not at chromosome ends (*NPS2*, *PKS5*, and *PKS7*) (Fig. 9), no products are known for the latter two PKSs, but note that NPS2 (ferrichrome synthetase), the most conserved of all fungal NRPSs and responsible for the critical task of chelating iron and protecting the cell from reactive oxygen species (Oide et al., 2007), is internally situated. However, *NPS6*, encoding an NRPS for extracellular siderophore biosynthesis, which is important in virulence and in resistance to low-iron and oxidative stress, is in a high-SNP/high-recombination region at the proposed fusion junction, as are the conserved genes corresponding to *C. heterostrophus* NPS4 (which has a conserved role in cell surface properties) and NPS10 (colony morphology and oxidative-stress resistance) (Fig. 9). Of these, only NPS10 is within the 300 kb of a supercontig end and not in an internal location suggested to be a chromosome fusion spot. PKS15, unique to *F. graminearum* and expressed specifically in planta (Gaffoor et al., 2005), is within 300 kb of the end of supercontig 3.2.

It is concluded that where data are available, core NPS and PKS genes have a telomere location bias.

Horizontal Transfer

Figure 2 is a cartoon of a genealogy constructed using the highly conserved PKS ketosynthase domain (Kroken et al., 2003). Before this work, the diversity and spotty distribution of *PKS* genes found in filamentous fungi had led to speculation, including our own (Rose et al., 2002; Yang et al., 1996), that horizontal gene transfer was involved (see Walton, 2000, and references therein). It is well documented that horizontal transfer and clustering of secondary metabolite pathways occur in bacteria (Kers et al., 2005; Lawrence, 1999; Lawrence and Ochman, 2002); thus, this early assumption to explain the discontinuous distribution of core *NPS* and *PKS* genes in fungal genomes had a precedent. Kroken et al. (Kroken et al., 2003) were the first to report genome-wide analyses of PKSs; observations supported the view that horizontal gene transfer among fungi does not need to be invoked to account for the highly discontinuous gene distribution and that gene duplication, divergence, and gene loss could explain this pattern. Bacterial and fungal PKSs grouped separately, with a few exceptions (*ChPKS24*, a hybrid *NPS:PKS*) that were possibly the result of horizontal gene transfer from bacteria to fungi. Fungal PKSs fell into eight groups: four are predicted to synthesize linear, reduced PKs, and four are predicted to produce aromatic, unreduced PKs. The phylogenomic distribution is consistent with the hypothesis that all eight types of *PKS* gene were present in the common ancestor of the four classes of the Pezizomycotina, before the radiation of Pezizomycotina 300 to 700 million years ago (Heckman et al., 2001; Kroken et al., 2003).

Figure 1 represents a genealogy constructed using A domains of NRPSs from 45 genomes (Bushley and Turgeon, unpublished). A subset of these was published previously (Lee et al., 2005) (see Fig. 7). It is clear, as with the PKSs, that fungal and bacterial NRPSs group separately, with the exception of the monomodular NRPSs at the top of the tree. Most clades below the siderophore clade (Fig. 1) are multimodular and found exclusively in Ascomycetes. Also in this group are representatives that show the most sporadic distribution. An exception is the NRPS/PKS clade (Fig. 1), which is mostly bacterial, except for the hybrid NRPS/PKS (NPS7/PKS24) from *C. heterostrophus*, mentioned above. To date, this fungus is the only one known to carry an N-terminal NRPS. In contrast, the PKS/NRPS clade at the top of the diagram in Fig. 1 has representatives from many ascomycetes. One of the few reasonably convincing cases for transfer of secondary metabolite clusters from bacteria to fungi is the ACV synthetases (Brakhage et al., 2005; Ullan et al., 2002), which biosynthesize linear tripeptide β-lactam antibiotics, including penicillin and cephalosporin (Fig. 1).

Horizontal transfer of clusters between fungi has been invoked as a mechanism to explain the discontinous distribution of secondary metabolite genes or pathogenicity gene clusters such as the *Nectria hematococca* pea pathogenicity gene cluster, which resides on a dispensible chromosome (Rodriguez-Carres et al., 2008; Temporini and VanEtten, 2004). Other proposed instances of horizontal transfer in fungi include the transfer of a virulence gene, *ToxA*, to

Pyrenophora tritici-repentis from *Stagonospora nodorum* (Friesen et al., 2006). A few studies have provided evidence for the horizontal transfer of clusters or partial clusters of secondary metabolite genes (Khaldi et al., 2008; Patron et al., 2007). Both the incongruence of phylogenies of individual genes within a cluster with an accepted species phylogeny, and the disjunct distribution of different subtypes of clusters even among closely related taxa, provide evidence for a horizontal gene transfer scenario. However, both of these studies demonstrated that there is a core set of genes common to all clusters and that all ACE1 (Khaldi et al., 2008) and all ETP-type clusters (Fig. 4) (Patron et al., 2007) most likely derive from a single ancestral cluster already assembled from the core set of genes in the ancestor of ascomycetes. These data argue against the independent evolution of clusters in distinct taxa and instead suggest a pattern of frequent loss of gene clusters in agreement with the studies by Kroken et al. (Kroken et al., 2003) and Lee et al. (Lee et al., 2005).

Consider the case of *C. heterostrophus* genes for T-toxin production and the *D. zeae-maydis* genes for PM-toxin production (Fig. 6). *C. heterostrophus* race T appeared suddenly in the field, concurrent with the southern corn leaf blight (SCLB) epidemic in 1969–1970; high virulence on T-cytoplasm corn is inseparable from the ability of race T to produce T-toxin. The *PKS1* gene for T-toxin production has no counterpart in *C. heterostrophus* race O, or in any other related species (Baker et al., 2006; Rose et al., 2002). Because *D. zeae-maydis* (also first identified at the time of the SCLB epidemic) PM-toxin structure is similar to that of T-toxin and the biological specificity is identical, we surmised that the same *PKS1* gene would be found in *D. zeae-maydis* and that one likely acquired it from the other (Turgeon and Baker, 2007). While it is true that the *DzmPKS* responsible for PM-toxin production is the closest match to *ChPKS1* for T-toxin, they are only 60% identical (Fig. 6C). The only firm conclusion that one can make is that neither fungus could have transferred this gene to the other at the time of the SCLB epidemic because the percent similarity is too low for a recent transfer. Furthermore, as noted earlier (see "Conserved versus Discontinuous Distribution" above), the *C. heterostrophus* T-toxin "clusters" (two loci on two different chromosomes) are unlike the apparently single PM-toxin cluster, and only the *PKS1* gene is in common (Turgeon and Baker, 2007).

Core *PKS* and *NPS* genes in fungal secondary metabolite clusters generally show GC content, introns (where present), and codon biases characteristic of other fungal, not bacterial, genes. Caution is warranted in invoking horizontal gene transfer as a mechanism to explain the distribution of secondary metabolite genes in fungi.

Is There an Association of Core PKSs or NPSs with Lifestyle?

When one considers the fungal lifestyle—pathogens versus saprobes, plant pathogens versus animal pathogens, root pathogens versus leaf pathogens, asexual versus sexual species, endophytes versus pathogens, heterothallic versus homothallic, etc.—are there factors that distinguish one lifestyle type from another or a factor that all plant pathogens, for example, share? The short answer appears to be no. Consider first the observation that few core genes for secondary metabolism are conserved across fungi and that most are irregularly distributed. The *C. heterostrophus NPS6* gene (Oide et al., 2006) is widespread and might be a candidate. Although the yeasts and filamentous basidiomycetes lack the corresponding gene, most filamentous ascomycetes carry it and require the corresponding product, an extracellular siderophore metabolite, for iron chelation under low-iron stress conditions and for resistance to oxidative stress. For plant pathogens in which NPS6 has been deleted, virulence on their respective host is reduced, as is resistance to both stress conditions (Oide et al., 2006). This factor is also important for *A. fumigatus* pathogenicity in mouse (Schrettl et al., 2007). It is missing apparently from the genome of the endophyte *E. festucae*, which has a novel NRPS for extracellular fusarinine production whose absence affects the harmonious relationship of the endophyte with the plant (Johnson, 2008). The argument that NPS6 is restricted to pathogens is untenable, since the saprobe *N. crassa*, for example, has this gene (and it can complement and restore a *C. heterostrophus nps6* deletion strain to full virulence on maize [Oide et al., 2006]). As the NPS6 metabolite is an extracellular siderophore, it makes sense that a high-affinity iron chelator would be important in the microbe-host interaction competition, as well as in other niches where iron is limiting. Thus, many ascomycetes employ this factor for iron gathering, but this ability is not associated with a particular lifestyle, as saprobes and plant and animal pathogens all use it. The association is with nutrient status, not with pathogenic potential. If we put aside NRPSs that synthesize siderophores and PKSs that produce melanin, are any metabolites specific to a certain lifestyle? One might be reminded of the fact that HSTs are key factors required for plant pathogenicity/virulence. However, each HST is a unique metabolite, made by a particular species or race, that acts as a virulence/pathogenicity factor on a particular host. All HSTs that we know about are produced by taxa in the Dothideomycetes, most notably by *Cochliobolus* and *Alternaria* species in the Pleosporaceae (Turgeon and Lu, 2000). Friesen et al. (Friesen et al., 2008) suggest that this group may be adept at taking up laterally transferred DNA. There is no compelling evidence for this, although we acknowledge that we have previously proposed this idea for *Cochliobolus* spp. (Turgeon and Baker, 2007; Turgeon et al., 1995).

Regulation

As noted above, many secondary metabolite gene clusters include pathway-specific transcription factors that, when deleted, diminish metabolite production (see "Are Core PKS and NPS Genes for Secondary Metabolism Often in Clusters?" above). A major breakthrough in understanding how expression of genes in clusters associated with secondary metabolism is regulated was the discovery that the *A. nidulans laeA* gene encodes a "global" regulator (Bok and Keller, 2004; Bok et al., 2006; Perrin et al., 2007; Shwab et al., 2007; Shwab and Keller, 2008). Absence of LaeA reduces expression of genes in a number of clusters, while overexpression causes an increase in production of the corresponding metabolites. A recent publication reports a more comprehensive regulatory picture (Bayram et al., 2008) by tying LaeA to the VelB/VeA complex, and thus coupling, mechanistically, light signaling to secondary metabolism and development (Bayram et al., 2008). It is known that light inhibits secondary metabolism and sexual reproduction in *A. nidulans* and that VeA is primarily expressed in the dark and interacts with VelB, which is expressed during sexual development. Deletion of *velB* or *veA* results in defects in both sexual fruiting-body formation and the production of secondary metabolites. In addition, expression and function of some secondary metabolite gene clusters have been shown to involve histone deacetylases (Shwab et al., 2007); this suggests that chromatin remodeling via histone modification might be a general regulatory mechanism.

Whether this control scenario is true for other fungi is unknown at present. For *C. heterostrophus*, it is clear that light does not inhibit T-toxin or siderophore production. The *laeA*-deletion strain is reduced in virulence and sensitive to oxidative stress, and preliminary data suggest that these phenotypes are not complemented by *A. nidulans laeA* (Zhang and Turgeon, unpublished). In addition, if *C. heterostrophus NPS2* is deleted in both mating partners, fruiting bodies are formed, but no ascospores are produced (Oide, 2007). This connects secondary metabolism to development; however, this phenotype is not sensitive to light conditions.

KNOWN NATURAL FUNCTIONS OF FUNGAL SECONDARY METABOLITES

Antibiosis

A number of secondary metabolites of fungal origin show activities contributing to protection from, and/or competition with, other organisms (bacteria, fungi, plants, insects, animals, etc.) (Degenkolb et al., 2008). Best known among these are the biocontrol activities of *Trichoderma* species (Benitez et al., 2004). Fungi-consuming insects avoid fungal sclerotia because of toxic secondary metabolites present in these structures (Demain and Fang, 2000; Gloer et al., 1988; Joshi et al., 1999). Aflavinines, indole diterpenoids produced by *Aspergillus flavus*, are present in sclerotia and inhibit feeding by the *Carpophilus hemipterus* beetle, which consumes all the other parts of the fungus (Gloer et al., 1988). Aflavinines have been identified as an anti-insectan also in the sclerotioid ascostromata of the species of *Eupenicillium* (Wang et al., 1995). The fruiting body of *Lentinellus ursinus* contains a terpenoid, isovelleral, which shows antifeedant activity against the opossum *Didelphis virginiana* (Camazine, 1983). *Armillaria mellea* is known to produce toxic sesquiterpenoids, such as armillyl orselimnate (Donnelly et al., 1982) and arnaminol (Donnelly et al., 1986), to eliminate the bacterial predators of its fruiting body (Abraham, 2001). The dung of herbivores is an attractive habitat for diverse species of coprophilous fungi, which appear to have adapted to this specific niche by evolving mechanisms to compete with other fungi (Gloer, 1995). *Stilbella erythrocephala* (syn. *S. fimetaria*) produces antifungal peptaibols antiamoebin I, III, and XVI in its natural substrate (rabbit dung), as well as in synthetic media (Lehr et al., 2006). Another example of niche competition through antibiosis is observed in mutualistic associations of fungi with plant hosts. Species of *Epichloe* and their asexual relatives *Neotyphodium* are endophytes of C3 grasses. These endophytic fungi are known to produce bioactive alkaloids such as peramine and lolines in planta (Clay and Schardl, 2002; Saikkonen et al., 2004). Protection of plant hosts from animal and insect herbivores by these fungal secondary metabolites has been well documented (Tanaka et al., 2005; Yue et al., 2000).

Toxins

The enniatins (Fig. 1), cyclohexadepsipeptides produced by species of *Fusarium*, affect leaf and root development of germinating wheat seeds (Burmeister and Plattner, 1987) and induce necrosis of tomato leaves and potato tubers (Herrmann et al., 1996a, 1996b). The bicyclic sesquiterpenoid botrydial, produced by *B. cinerea*, has been implicated as a strain-specific virulence determinant (Colmenares et al., 2002; Deighton et al., 2001, Siewers et al., 2005). In some cases, i.e., HSTs, biosynthesis of phytotoxins is essential for fungal pathogenicity (Walton and Panaccione, 1993; Wolpert et al., 2002), and loss of ability to biosynthesize the HST results in avirulence (Johnson et al., 2000; Scheffer et al., 1967). For example, the cyclic depsipeptide AM-toxin, the cyclic tetrapeptide HC-toxin, and the cyclic chlorinated pentapeptide victorin are all essential for infection by their producing fungi on their susceptible hosts (Johnson et al., 2000; Walton, 1996). The virulence activities of *C. heterostrophus* T-toxin and *D. zeae-maydis* PM toxin have been described exhaustively (Turgeon and Baker, 2007; Turgeon and Lu, 2000).

Roles for toxic secondary metabolites in fungal virulence to animal hosts have not been explored as extensively as have roles in fungal virulence to plants. Siderophores produced by the animal pathogen *A. fumigatus* play a role in infection of mice (Grovel et al., 2002; Nieminen et al., 2002). Whether or not these siderophores act as a toxin, however, is undetermined. Siderophores could show toxicity by depleting iron in host cells. On the other hand, they could also help fungal infection as a source of iron in hosts. It is important to point out that these two proposed roles of siderophores are not mutually exclusive. Gliotoxin, produced by *A. fumigatus*, is associated with cytotoxicity (Suen et al., 2001) and apoptotic properties (Bok et al., 2006a; Kweon et al., 2003); however, evidence for a role in virulence is contradictory (Cramer et al., 2006; Spikes et al., 2008; Sugui et al., 2007). Apart from the studies on gliotoxin, there is no clear demonstration of the role of toxic secondary metabolites in fungal infection of animal hosts.

Development

An association between secondary metabolite biosynthesis and fungal development has long been noted. For instance, a diterpenoid, conidiogenone, isolated from *Penicillium cyclopium* is an inducer of asexual sporulation (Roncal et al., 2002). The intracellular siderophore NPS2 is critical for meiosis in heterothallic *C. heterostrophus* and homothallic *G. zeae* (Oide, 2007) and for both asexual and sexual development of *A. nidulans* (Eisendle et al., 2006). *M. oryzae* ACE1 expression is correlated with initiation of appressorium-mediated penetration into the plant (Fudal et al., 2007), and the NRPS is localized in the appressorium. Recently, an NRPS metabolite has been extracted from spores of the invertebrate pathogen *Metarhizium anisopliae*; however, no developmental phenotype has been determined (Moon et al., 2008).

A. nidulans psi factors (oxylipins) induce sexual development while inhibiting asexual development (Champe et al., 1987). Psi factors, in fact, can be inducers of both sexual and asexual sporulation, and their role appears to be to regulate the ratio of asexual to sexual spores in *A. nidulans* (Champe et al., 1987). A proposed secondary metabolite (butyrolactone I) induces asexual sporulation in *Aspergillus terreus* (Schimmel et al., 1998). Generally, fungal spores do not germinate efficiently when spore density is high. Self-inhibition of asexual spore germination has been reported for fungal and oomycete species from diverse genera, including *Peronospora* (Leppik et al., 1972), *Penicillium* (Chitarra et al., 2004), and *Puccinia* (Macko et al., 1970). *Colletotrichum graminicola* forms asexual spores within an acervulus in which spores are bound by a water-soluble, mucilaginous matrix (Nicholson and Epstein, 1991). Inhibitory activity of spore germination was observed in the spore mucilage; a compound responsible for this activity was identified as mycosporine-alanine (Leite and Nicholson, 1992), and this metabolite is proposed to prevent germination of spores in acervuli before dispersal (Gottlieb, 1973). Although these compounds are not the

product of NRPS or PKS biosynthesis, they illustrate possible roles for NRPS and PKS metabolites. Along these lines, a recent publication reported that the polyketide citrinin, produced by *Penicillium citrinum*, induces swarming motility of *Paenibacillus polymyxa*, a soil bacterium (Park et al., 2008). Flagellum staining showed that citrinin activated the production of flagella by the bacterium. While the biological significance of this is unclear, it is not hard to imagine that some secondary metabolites act as signaling molecules for the fungi themselves.

Metal Metabolism

Siderophores are secondary metabolites with strong iron-chelating activity. Extracellular siderophores contribute to acquisition of iron, which is usually limited in aerobic environments. Intracellular siderophores show antioxidant properties by preventing iron-catalyzed generation of cytotoxic free radicals. In bacteria, interactions between secondary metabolites and metals are not restricted to iron-siderophore complexes. In the methane-oxidizing bacteria, copper plays a central role in metabolism, in contrast to most other bacteria, in which iron is the central player (Kim et al., 2004). Hence, the methane-oxidizing bacteria have been postulated to have unique copper-trafficking systems, in order to fulfill requirements for copper (Choi et al., 2003). One study reported isolation of a copper-chelating siderophore analog, methanobactin, from the methane-oxidizing bacterium *Methylosinus trichosporium* (Gaspar et al., 1999). Whether or not methanobactin actually plays a role in copper-trafficking of this species has not yet been examined. However, the study demonstrated the presence of a siderophore analog of microbial origin, which shows strong binding activity to a metal other than iron. In another report, an artificially synthesized hydroxamate siderophore analog showed chelating properties to copper and aluminum, as well as to iron (Gaspar et al., 1999). Taken together, these results imply that microbial secondary metabolites may be involved in metabolism of metals other than iron. As noted earlier (see "Conserved versus Discontinuous Distribution" above), we have recently discovered that the *ChNPS1* gene is activated by Zn levels (Zhang and Turgeon, unpublished).

Abiotic Stress

Some fungal secondary metabolites contribute to stress tolerance of the producers. The role of DHN-melanins (whose biosynthesis involves a PKS) in protection against UV irradiation has been reported in different species of fungi (Kawamura et al., 1999; Leonard, 1977). Spores of albino mutants of *A. alternata* are more sensitive to UV light than spores of wild-type strains (Kawamura et al., 1999). Antioxidant properties of melanins have also been reported for *A. alternata* and *Exophiala dermatitidis* (Jacobson et al., 1995). In some species, melanins are proposed to play a role in tolerance against extremes of temperatures (Jumpponen and Trappe, 1998). Melanins have also been reported as a virulence determinant in some plant pathogenic fungi, such as *Colletotrichum lagenarium* (Takano et al., 1995) and *M. oryzae* (Howard and Valent, 1996).

SUMMARY

The diversity of small-molecule metabolites produced by fungi is staggering. We contend that these small molecules are the currency of day-to-day affairs of fungal cells. Not only do they broker associations with other organisms, but also they play fundamental roles in morphological and reproductive development, nutrient gathering, and stress management.

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Plant Cell Wall and Chitin Degradation

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Fungi are heterotrophic organisms and consequently depend on the utilization of preformed organic matter as a source of energy for cellular biomass formation. They have acquired an astonishing ability for the degradation of all sorts of organic compounds ranging from simple low-molecular-weight monomers to complex high-molecular-weight macromolecules. Today, they serve as the most important and widespread group of organisms performing the recycling of plant material and are therefore key players of the global carbon cycle. A large group of fungi has thereby specialized in the degradation of the complex plant cell walls. Saprotrophic or saprobic fungi, which grow on dead plant materials, are particularly important for this process, yet fungi that have evolved as necrotrophic and biotrophic parasites or pathogens of living plants also substantially contribute to degradation. This is particularly important for several agriculturally important crops that are attacked by fungi. Plant pathogenic fungi lead to substantial crop losses each year, either by decomposing plants or by producing toxic substances, which renders the crops inedible for human consumption.

There are several reasons why researchers have worked in the field of plant cell wall degradation by fungi. One is the attractive potential to use the fungal plant cell wall-degrading enzymes in biotechnological applications, ranging from biomass hydrolysis for the production of biofuel or fine chemicals to the improved manufacture of food and feed, textiles, and paper. Due to the increasing shortage of fossil raw materials, the production of bioethanol from renewable raw material and especially the potential of plant cell wall-degrading enzymes to convert lignocellulosic biomass are currently of great interest. The natural resistance of plant cell walls to microbial and enzymatic decomposition is largely responsible for the high cost of lignocellulose conversion. To achieve sustainable energy production, it will be necessary to overcome the chemical and structural properties that have evolved in this renewable biomass to prevent its disassembly (Himmel et al., 2007).

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A polymer that is structurally related to the plant cell wall polysaccharide cellulose but does not occur in plants is chitin. Being the main compound of invertebrate exoskeletons and also an essential structural component of the fungal cell walls of members of Ascomycota and Basidiomycota, it constitutes the second most abundant polymer found in the biosphere after cellulose. It is therefore not surprising that fungi also have the ability to degrade and metabolize this polymer. Although it does not yet rival lignocellulose in its biotechnological application, interest in enzymatic hydrolysis of chitin from shellfish waste and the application of its monomers is increasing, particularly in the pharmaceutical area.

Fungi produce a broad range of mainly extracellular enzymes to degrade the various cell wall polymers. Numerous genes were cloned, and many proteins have been identified within the last century. Nevertheless, recent genomic approaches to unravel the sequences of ascomycetous and basidiomycetous fungi demonstrate that many additional genes encoding putative cell wall-degrading enzymes are present in these genomes. A classification of these and other nonrelated carbohydrate active enzymes based on sequence homology can be found online in the CAZy database (http://www.cazy.org).

STRUCTURE AND COMPOSITION OF PLANT CELL WALLS AND PRINCIPLES OF THEIR DEGRADATION

The polymers that fungi encounter when they are in contact with living or dead plants are cellulose, hemicelluloses, and pectins. Together with the aromatic polymer lignin and proteins, these compounds form a complex and rigid structure, lignocellulose. From the point of view of practical application, most strategies to use plant cell walls in biotechnological processes exploit the cellulose and hemicellulose sugars following depolymerization. The heterogeneous lignin polymer is problematic, as it blocks access to these usable polymers. One emerging approach is to genetically engineer plants to be defective in lignin biosynthesis to make sugars more available and increase fuel yields per unit biomass (Chen and Dixon, 2007). Often the remaining lignin is burnt to provide energy for the processing factory.

In plant cells, the middle lamella, the primary wall, and the secondary wall are the three major regions to be distinguished. The middle lamella is the outermost layer of the plant cell and is primarily composed of pectin. The primary wall expands inside this middle lamella and consists of a matrix composed of polysaccharides and (glyco)proteins. The cellulose microfibrils in the primary wall are aligned at all angles and cross-linked via hemicellulosic tethers (e.g., xyloglucan and galactoglucomannan) to form the cellulose-hemicellulose network, which is embedded in the gelatinous pectin matrix (homogalacturonan, rhamnogalacturonan I [RG-I], and rhamnogalacturonan II [RG-II] (O'Neil and York, 2003). Primary cell walls can be grouped into two types (Carpita and Gibeaut, 1993). Type I is found in all dicotyledons, nongraminaceous monocotyledons, and gymnosperms and contains typically xyloglucan and/or glucomannan and 20 to 35% pectin. Type II is found in the monocotyledons of the Poaceae family and is rich in arabinoxylan but contains <10% pectin.

A secondary cell wall is found in many plant cells, located between the primary cell wall and the plant cell membrane. When the primary cell wall is complete and cell growth has stopped, a secondary cell wall, which primarily consists of cellulose and lignin, is formed in wood. In addition, the secondary cell wall can contain phenols, tannins, lipids, sterols, sugars, and proteins.

The polysaccharides found in the cell wall are either linear polymers composed of a single type of glycosyl residue (e.g., cellulose is composed of 1,4-linked β-glucosyl residues), polymers with a regular branching pattern (e.g., xyloglucan and RG-II), or as in the case of RG-I, substituted with a diverse range of arabinosyl- and galactosyl-containing oligosaccharide side chains.

PHYSIOLOGICAL AND ECOLOGICAL IMPLICATIONS OF PLANT CELL WALL DEGRADATION

Most of the plant cell wall polysaccharides occur in the form of lignocelluloses. Their structural and chemical complexity, as outlined above, usually necessitates the concerted action of several microorganisms for effective degradation, in which fungi mostly play the dominant role. Fungi found in these habitats live in complex and diverse communities and follow generalized decomposition sequences. That these fungi are often specialized for the degradation of only some of the components is reflected by the number and diversity of genes encoding polymer-degrading enzymes in their genomes. The regulation of expression of genes encoding plant cell wall-degrading enzymes is controlled by sophisticated mechanisms, and highly productive secretory machineries provide export to the environment. Taken together, these characteristics make fungi successful competitors with other microorganisms, and fungi are thus the key players during decomposition and nutrient cycling in terrestrial and aquatic ecosystems.

In the case of wood, three different types of degradation can be distinguished: white rot, brown rot, and soft rot. White rots are the major group of wood-rotting fungi and the only group that can completely degrade lignin to CO_2 and H_2O. They also overcome intrinsic bottlenecks of wood degradation, such as its low nitrogen content (C/N, ~500) and the presence of growth-inhibitory compounds. Lignin and (hemi)cellulose can be either simultaneously or sequentially degraded by white-rot fungi. During simultaneous attack, which occurs in hardwood, the cell wall is progressively attacked from the cell lumen towards the middle lamella, degradation being exclusively associated with the fungal hyphae. Since enzymes are usually too large to enter the plant cell wall, degradation occurs only in exposed regions of the cell lumen. This type of mechanism has been observed mainly for basidiomycetes (e.g., *Trametes versicolor*, *Irpex lacteus*, *Phanerochaete chrysosporium*, *Heterobasidion annosum*, and *Phlebia radiata*) and some ascomycetes (e.g., *Xylaria hypoxylon*). In the sequential attack that occurs with basidiomycetes such as *Ganoderma australe*, *Phlebia tremellosa*, *Ceriporiopsis subvermispora*, *Pleurotus* spp., and *Phellinus pini*, the initial attack selectively targets lignin and hemicellulose and starts at the middle lamella and secondary wall. Cellulose is only attacked thereafter. It should be noted, however, that many white-rot fungi can use both types of rot, depending on the substrate (Eriksson et al., 1990; Martinez et al., 2005).

The term "brown rot" refers to the reddish brown appearance of the resulting decayed wood. Brown-rot fungi extensively degrade wood but attack only the (hemi)cellulose part and not the lignin. Due to cellulose degradation, brown rot rapidly leads to a loss of mechanical strength and leaves the oxidized lignin, which cracks to form characteristic brick-like pieces. Representatives of brown-rot basidiomycetes are *Schizophyllum commune*, *Fomes fomentarius*, *Serpula lacrymans*, and *Gloeophyllum trabeum*. They are also the major cause of decay of wood products and have an important role in coniferous ecosystems through their contribution to humus formation.

Some ascomycetes can also colonize wood but modify lignin only weakly. Their action leads to a decrease in the mechanical properties of wood, giving rise to so-called soft rot. Soft-rot fungi can also degrade wood under extreme environmental conditions (extreme wetness or frequent dryness) that prohibit the activity of other wood-degrading fungi. Soft-rot fungi (such as *Chaetomium*, *Ceratocystis*, and *Phialophora)* are unspecialized (hemi-)cellulolytic ascomycetes. Two distinct types of soft rot are currently recognized. Type 1 is characterized by longitudinal cavities formed within the secondary wall of wood cells, and type 2 is characterized by an erosion of the entire secondary wall (Martinez et al., 2005).

CELLULOSE AND HEMICELLULOSE DEGRADATION

Cellulose constitutes the most abundant biopolymer on earth, comprising about 50% of the renewable biomass with a formation rate of 4×10^9 tons annually. It is a linear and highly ordered polymer that consists of about 8,000 to 12,000 β-1,4-linked D-glucopyranose units. In contrast to the homopolymer cellulose, hemicelluloses are heterogeneous and account for around 20 to 30% of plant cell wall biomass (Eriksson et al., 1990). These plant cell wall polysaccharides have a backbone of 1,4-linked β-D-pyranosyl residues, which may be xylosyl, glucosyl, galactosyl, arabinosyl, or mannosyl residues, which give rise to their generic names, such as xylans. The enzymes involved in the degradation of cellulose and hemicellulose are classified as endoacting enzymes, which attack from a random point along the polymer chain, and exoacting enzymes, which release monomers or dimers from the chain ends. The higher chemical diversity of hemicelluloses requires a larger set of enzymes that can act either on the main or side chains. A common characteristic of all these enzymes is that they act synergistically to hydrolyze the polysaccharides.

Cellulose Degradation by White-Rot and Soft-Rot Fungi

The complete hydrolysis of cellulose to D-glucose requires the cooperation of three enzymes: cellobiohydrolases, endoglucanases, and β-glucosidases. Cellobiohydrolases (EC 3.2.1.91; 1,4-β-D-glucan cellobiohydrolases) act processively from the ends of the cellulose chain to generate mainly the glucose disaccharide cellobiose. Endoglucanases (EC 3.2.1.4; 1,4-β-D-glucan 4-glucanohydrolase) cleave the cellulose chains internally in the amorphous regions and in this way generate additional sites for the cellobiohydrolases to degrade the cellulose. β-Glucosidases (EC 3.2.1.21) degrade the accumulating products (oligosaccharides and cellobiose) of the cellobiohydrolase and endoglucanase to D-glucose (Beguin, 1990; Teeri, 1997). Cellobiohydrolases belong to glycoside hydrolase (GH) families 6 and 7. GH 6 enzymes cleave from the nonreducing chain end and act via an inversion mechanism, resulting in α-anomeric products. In contrast, GH 7 enzymes attack from the reducing end and have a retaining mechanism with β-anomers being produced. Fungal endoglucanases mainly belong to GH families 45 and 61, whose catalytic mechanism has not yet been extensively studied.

Most fungi are able to utilize cellulose as a carbon source, although not all of them can utilize cellulose as the sole carbon source, which is probably due to differences in the regulation of cellulase formation (see below). Cellulase-encoding genes have been isolated from a large number of asco- and basidiomycetes. The best studied cellulolytic fungus is *Trichoderma reesei* (the anamorph of the pantropical fungus *Hypocrea jecorina*), which was discovered as a strong degrader of cellulosic material by the U.S. Army on the Solomon Islands during World War II (Kubicek and Penttilä, 1998). However, the cellulolytic systems of other fungi, including various *Aspergillus* and *Humicola* spp. and the white-rot fungus *Phanerochaete chrysogenum*, have also been investigated (Aro et al., 2005; de Vries, 2003).

An important feature of many cellulases—and also of other glycoside hydrolases—is the presence of a carbohydrate-binding domain. These domains are classified into families of carbohydrate binding modules (CBMs) based on their amino acid sequences. The annotation of fungal genomes has resulted so far in identification of more than 300 putative CBMs. For more details, see the Carbohydrate-Binding Module Family Server: http://www.cazy.org/fam/acc_CBM.html. The carbohydrate-binding domain of cellulases, originally described as a cellulose-binding domain, has been reclassified as CBM 1. A role for CBM 1 in binding to other polymers such as chitin has been the subject of recent reviews (Boraston et al., 2004; Shoseyov et al., 2006).

CBMs are about 40 amino acids in size, are located at the N or C terminus of the protein, and are spatially separated from the catalytic enzyme domain by an unstructured, often O-glycosylated linker region that enables flexibility in binding and catalysis. The biological functions of CBMs involve targeting of an enzyme to its substrate and maintaining proximity of the enzyme with its substrate. In addition, since some CBMs have the ability to disrupt the crystalline polysaccharide structure, removal of the CBM impairs the activity against insoluble substrates.

The first cellulase for which a crystal structure was determined was the catalytic domain of *T. reesei* cellobiohydrolase II (Rouvinen et al., 1990). It has a tunnel-shaped active site that is so narrow that there is only space for a single cellulose chain, and therefore, it can attack only from the ends of the chain. In contrast, although endoglucanases are similar in the overall three-dimensional structure, subsequent studies showed that they have a wider active site, which allows the attack of the cellulose chains from any random point.

In addition to cellulase enzymes, several novel types of proteins that are also involved in cellulose degradation have been described. Most intriguing in this regard are the swollenins, which show similarity to plant expansins. Expansins induce the extension of isolated cell walls (McQueen-Mason et al., 1992) and exhibit nonhydrolytic disrupting activity on cell wall polymers, e.g., pectins and xyloglucans, which are tightly bound to the cellulose microfibrils (McQueen-Mason and Cosgrove, 1995). Swollenins do not exhibit any hydrolytic activity but are able to disrupt the cellulose microfibrils and thus render the cellulose fibers more accessible for attack by cellulases (Saloheimo et al., 2002). *H. jecorina* SWO1 is composed of an N-terminal CBM1, which is connected by a linker region to the expansin-like domain. In addition, SWO1 contains a region similar to mammalian fibronectin type III repeats, which has not been identified in other fungal proteins.

Cellulose Degradation by Brown-Rot Fungi

Brown-rot fungi use a completely different method of cellulose degradation in comparison to white-rot and soft-rot fungi. Although their degradation process is still not entirely understood, most brown-rot fungi lack cellobiohydrolases (i.e., the ability to hydrolyze crystalline cellulose enzymatically) and instead use a synergism of oxidative attack and endoglucanases. Reactive oxygen species such as hydroxyl radicals (·OH) and the less reactive peroxyl (ROO·) and hydroperoxyl (·OOH) radicals are believed to be the agents that initiate degradation (Hammel et al., 2002). The pathway for the generation of these radicals is thought to occur via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \cdot OH$). Since the hydroxyl radicals would also damage the fungal hyphae, their production must occur at a safe distance from the hyphae, and the responsible reductant should also be stable enough to diffuse this distance before reducing Fe^{3+} and O_2 to Fe^{+2} and H_2O_2. Most brown-rotters secrete oxalic acid, which is a strong chelator of Fe^{3+} and Fe^{2+} and also reduces the pH. The pH of wood itself is generally in the range of 3 to 6 and is lowered to pH values between 2.5 and 1.7 during the decomposition. This reduction of pH is critical for the function of the extracellular enzymes and has been attributed as a key factor in the process.

Various systems have been discussed to produce ·OH radicals for brown rot, including secreted hydroquinones, cellobiose dehydrogenases (CDHs), low-molecular-weight glycopeptides and phenolate chelators. Hydroquinones are extracellularly reduced and subsequently react with Fe^{3+} to give Fe^{2+} and a semiquinone radical. The semiquinone reduces O_2 to ·OOH, which is a source for H_2O_2, and is in this way recycled to quinone. *G. trabeum* produces such extracellular quinines, including 2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone, which can also be reduced back to hydroquinones, possibly by the action of an intracellular quinone reductase.

Another nonenzymatic system includes phenolate or catechole chelators, such as 4,5-dimethoxy-1,2-benzenediol and 2,5-dimethoxy-1,4-benzenediol, which were originally isolated from *G. trabeum*. They are characterized by a high affinity for iron and by their ability to reduce Fe^{3+} to Fe^{2+}. ·OH radicals can also be produced by the extracellular flavohemoprotein CDH, which has so far been identified in all wood-degrading fungi (Zamocky et al., 2006). CDHs can act as cellobiose oxidases, reducing O_2 to H_2O_2, but Fe^{3+} has turned out to be a far better electron acceptor than O_2. Therefore, CDHs are in fact Fe^{3+} reductases. Glycopeptides, implicated into wood degradation, have been isolated

from G. *trabeum* and *Tyromyces palustris*. They reduce Fe^{3+} to Fe^{2+}, bind Fe^{2+}, and generate one-electron oxidation in the presence of H_2O_2 (Enoki et al., 2003; Goodell, 2003).

Biodegradation of Hemicelluloses

Xylans

Xylans, a highly heterogeneous group of polymers, are characterized by a β-1,4-linked β-D-xylosepyranose backbone and include arabino-, glucurono-, and glucuronoarabinoxylans (Fig. 1). They are abundant in the walls of cereals (*Poaceae*) and in the secondary walls of woody plants (hardwood) (Darvill et al., 1980; Ebringerova and Hienze, 2000). The backbone can be decorated with α-1,2- or α-1,3-linked L-arabinofuranose residues (such as in cereals [Izydorczyk and Biliaderis, 1995]) or with α-1,2- and α-1,3-linked 4-O-methyl-α-D-glucuronic acid. L-Arabinose residues can further be esterified at O-5 with feruloyl or *p*-coumaroyl residues. In hardwoods, acetyl groups are found at O-2 or O-3.

Hydrolysis of the xylan backbone involves endo-1,4-β-xylanases (endo-1,4-β-D-xylan xylanohydrolases [EC 3.2.1.8]) and β-xylosidases (1,4-β-D-xylan xylohydrolase [EC 3.2.1.37]). Endoxylanases cleave the main sugar chain depending on the type of xylan, the degree of branching, and the presence of different substituents (Polizeli et al., 2005). The main hydrolysis products, consisting of substituted or nonsubstituted oligomers, are further hydrolyzed by β-xylosidases to tri-, di-, and monomers. Endoxylanases have been subdivided by their ability to release L-arabinose from arabinoxylan (Wong et al., 1988) into debranching and nondebranching enzymes. Genes encoding xylanases and β-xylosidases are found in all fungal genomes, and their proteins have been characterized in detail particularly from *Aspergillus*, *Trichoderma*, and *Penicillium* spp., as well as *Agaricus bisporus* or *Magnaporthe grisea*.

Xyloglucan

Xyloglucan is quantitatively the predominant hemicellulosic polysaccharide of dicotyledons and nongraminaceous monocotyledons, comprising up to 20% of the plant cell wall. It links the cellulose microfibrils together and supports the structural integrity of the cell wall. Its backbone is composed of β-1,4 linked β-D-glucopyranose residues, which are often substituted with D-xylopyranose via an α-1,6-linkage. Depending on the number of backbone residues substituted with xyloses (X), xyloglucans are classified in either the XXXG or the XXGG type, the latter occurring in solanaceous plants. Further residues of the side chains include L-fucopyranose, D-galactopyranose, L-galactopyranose, or L-arabinose residues or O-linked acetyl groups (Fig. 2) (O'Neil and York, 2003). The xyloglucan backbone does not necessarily require specific enzymes for hydrolysis, as several endoglucanases (see above) are also active on xyloglucans, but some specific xyloglucan hydrolases (EC 3.2.1.151) have been reported as well (Grishutin et al., 2004; Hasper et al., 2002; Pauly et al., 1999).

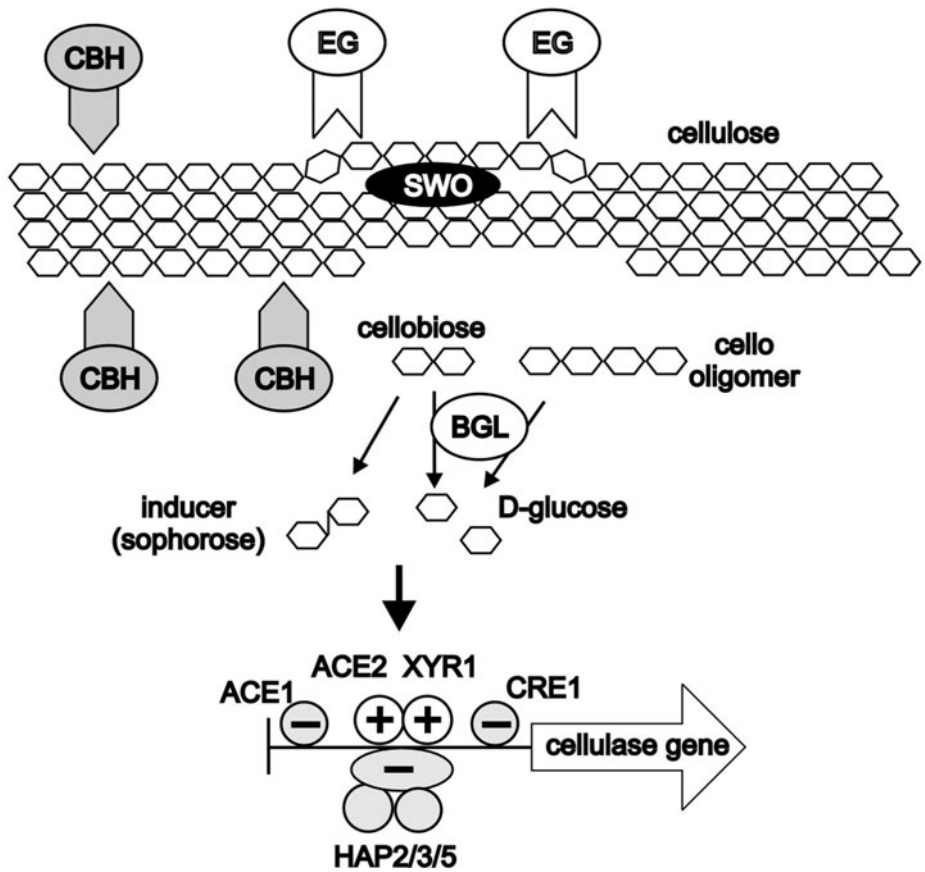

FIGURE 1 Schematic structure of major hemicelluloses in plant cell walls. Hemicelluloses consist of branched polysaccharides that have a backbone composed of 1,4-linked β-D-pentosyl/hexosyl residues. The predominant hemicellulose in many primary walls is xyloglucan, while the other hemicelluloses, including glucuronoxylan, arabinoxylan, arabinoglucuronoxylan, and galactoglucomannan, occur in both primary and secondary cell walls.

Galactomannans and Galactoglucomannans

Galactomannans and galactoglucomannans are the major hemicellulosic structures of softwoods, and glucomannans dominate in hardwood (Aspinall, 1980; Stephen, 1982). These mannans have a backbone of randomly distributed β-1,4-linked β-D-glucopyranose and β-D-mannopyranose residues, respectively. They are substituted with α-1,6-linked α-D-galactopyranose residues, which can further carry α-1,2-linked α-D-galactopyranose side chains. The D-glucose or D-mannose residues are partially acetylated at O-2 or O-3 (Fig. 2).

The backbone of galactoglucomannans is degraded by endo-1,4-β-mannanases (mannan endo-1,4-β-mannosidase [EC 3.2.1.78]) and β-mannosidases (EC 3.2.1.25) as described for xylans, in synergism with enzymes (α-galactosidases) removing the side chains.

Hemicellulosic Side Chains

Side groups in hemicelluloses are generally small (monomers to trimers) but can consist of several different sugars and acids (e.g., acetic acid, L-arabinose, ferulic acid, D-galactose, and D-glucuronic acid), and consequently, multiple enzymes are required to make the backbone fully accessible for the other hemicellulases. An overview of the enzymes involved in the release of the sugars and acids is given in Table 1.

Regulation of Fungal Cellulase and Hemicellulase Formation

Fungi show considerable versatility in utilizing a wide range of carbon sources, which helps them survive in a competing environment where the resources are limited. Degradation and catabolism of the individual carbon sources present in complex mixtures follow a mainly energy-driven hierarchy, but adaptation of saprobic and plant pathogenic fungi to their habitats has resulted in species-specific carbon source priorities. The main mechanisms, including carbon sensing and signaling, guarantee this assimilation hierarchy and lead to specific induction or general carbon catabolite repression. Fungi are particularly adapted for targeting and responding to D-glucose, the most abundant monosaccharide in nature (see chapter 21).

The regulation of the transcription of cellulase- and hemicellulase-encoding genes has been predominantly studied in *Aspergillus niger* and *T. reesei*. These studies revealed interesting similarities and differences between these two fungi (for a detailed review, see Aro et al., 2005; Schmoll and Kubicek, 2003; and Stricker et al., 2008). In both species, most of the respective enzymes are adaptively formed and their transcripts are not detectable during growth on readily available soluble carbon sources. Carbon catabolite repression, i.e., the prevention of formation of an inducible enzyme by D-glucose or any other carbon source that is an intermediate in catabolism, generally occurs more stringently in *A. niger* than in *T. reesei*. Expression of the majority of the cellulase genes that have been studied in filamentous fungi is repressed during growth on D-glucose. Glucose repression has been interpreted to override induction, since addition of D-glucose to induced cultures results in a lack of cellulase gene expression (Ilmen et al., 1997). This repression is not directly caused by binding of the major carbon catabolite repressor CRE1 to the (hemi)cellulase-encoding genes but rather is due to inducer exclusion. This is based on the evidence that carbon catabolite repression of hemi(cellulase) genes occurs still in *H. jecorina* RUT-C30, a mutant producing only a truncated form of CRE1, and that glucose inhibits the uptake of the cellulase-inducing disaccharide sophorose (Kubicek et al., 1993), thus leading to inducer exclusion.

Cellulase transcripts have also been detected in cultures of *T. reesei* in which D-glucose had already been consumed.

TABLE 1 Fungal glycoside hydrolases (GH) and carbohydrate esterases (CE) involved in the degradation of the side chains of plant cell wall polysaccharides

Enzyme	EC no.	Substrate	CAZy family
Exo-1,5-α-L-arabinanase	EC 3.2.1.–	Pectin	GH93
Endo-1,5-α-L-arabinosidase	EC 3.2.1.99	Pectin	GH43
α-L-Arabinofuranosidase	EC 3.2.1.55	Xylan, pectin, xyloglucan	GH3, 43, 51, 54, 62
Acetylxylan esterase	EC 3.1.1.72	Xylan	CE1, 2, 3, 5, 6
α-L-Fucosidase	EC 3.2.1.51	Xyloglucan, pectin	
α-1,2-L-Fucosidase	EC 3.2.1.63	Xyloglucan, pectin	GH95
Endo-β-1,4-galactanase	EC 3.2.1.89	Pectin	GH53
Endo-β-1,6-galactanase	EC 3.2.1.164	Pectin	
Exo-β-1,3-galactosidase	EC 3.2.1.145	Pectin	GH35
Feruloyl esterase	EC 3.1.1.73	Xylan, pectin	CE1
α-Galactosidase	EC 3.2.1.22	Xyloglucan, xylan, galacto(gluco)mannan, pectin	GH27, 36
β-Galactosidase	EC 3.2.1.23	Xyloglucan, xylan, pectin	GH2, 35
α-Glucuronidase	EC 3.2.1.139	Xylan, pectin	GH67
4-O-Methyl-glucuronoyl esterase	EC 3.1.1.–	Xylan	CE15
Pectin methylesterase	EC 3.1.1.11	Pectin	CE8
Pectin acetylesterase	EC 3.1.1.–	Pectin	
Rhamnogalacturonan acetylesterase	EC 3.1.1.–	Pectin	CE12
α-Rhamnosidase	EC 3.2.1.40	Pectin	GH78
Xylan α-1,2-glucuronosidase	EC 3.2.1.131	Xylan	GH67
α-D-Xylosidase	EC 3.2.1.–	Xyloglucan	GH31
β-1,4-D-Xylosidase	EC 3.2.1.37	Xylan, pectin	GH3, 43

But since incubation in medium lacking any carbon source did not induce cellulase transcription, it is likely that an inducing sugar is derived from carbohydrates released from the fungal cell wall or formed, e.g., by transglycosylation.

Consequently, most of the formation of cellulases and hemicellulases is due to specific induction. The mechanism by which insoluble carbon sources can trigger their own induction has been enigmatic for a long time, but it is now generally accepted that a first, limited degradation of the insoluble molecule must occur to form the respective inducers. Whether the inducer-producing cellulases are expressed at a low constitutive level, are present on the conidial surface, or are carbon catabolite derepressed is not yet known, and it is also possible that all three mechanisms occur under different growth conditions. In this regard, it is intriguing that genome sequencing and transcriptome analysis of *T. reesei* have identified a constitutively expressed endoglucanase (CEL5b) that contains a glycosylphosphatidylinositol anchor and is therefore probably attached to the plasma membrane. By analogy, the acetylxylan esterase AXE2, which is also predicted to contain a glycosylphosphatidylinositol anchor, could be involved in the induction of hemicellulases.

Various components have been shown to act as inducers of cellulases and hemicellulases in vitro, including soluble disaccharides resulting from their enzymatic attack (such as cellobiose, cellobiono-1,5-lactone, and xylobiose), transglycosylation products of these (such as sophorose or lactose), and also monomers (D-xylose, D-galactose, and L-arabinose) and the corresponding polyols. Their efficacy as inducers varies among different fungi and notably between *A. niger* and *T. reesei*: in *A. niger* D-xylose induces both cellulases and hemicellulases, but sophorose or cellobiose do not have any effect, whereas in *T. reesei* cellulases are induced only by sophorose, cellobiose, or lactose but not by D-xylose. This difference may reflect the different habitats of the two fungi and suggests that *A. niger* recognizes the availability of cellulose by the presence of hemicellulose monomers, whereas the enzymes of *T. reesei* are more specifically induced by cellulosic saccharides. The role that D-galactose and D-galactose-containing oligosaccharides play in the induction process of cellulases in *T. reesei* is also conspicuous, suggesting a specific niche in a D-galactose-rich environment.

Molecular details of cellulase and hemicellulase regulation in *A. niger* and *T. reesei* also illustrate the differences in regulation between these two ascomycetes: in *T. reesei*, at least three transcriptional activators, XYR1 (Stricker et al., 2006), ACE2 (Aro et al., 2001), and the HAP2/3/5 complex (Zeilinger et al., 2001), have been shown to be involved in specific induction, with XYR1 playing the dominant role (Fig. 1). In addition to the general carbon catabolite repressor CRE1 (Ilmen et al., 1996), a second

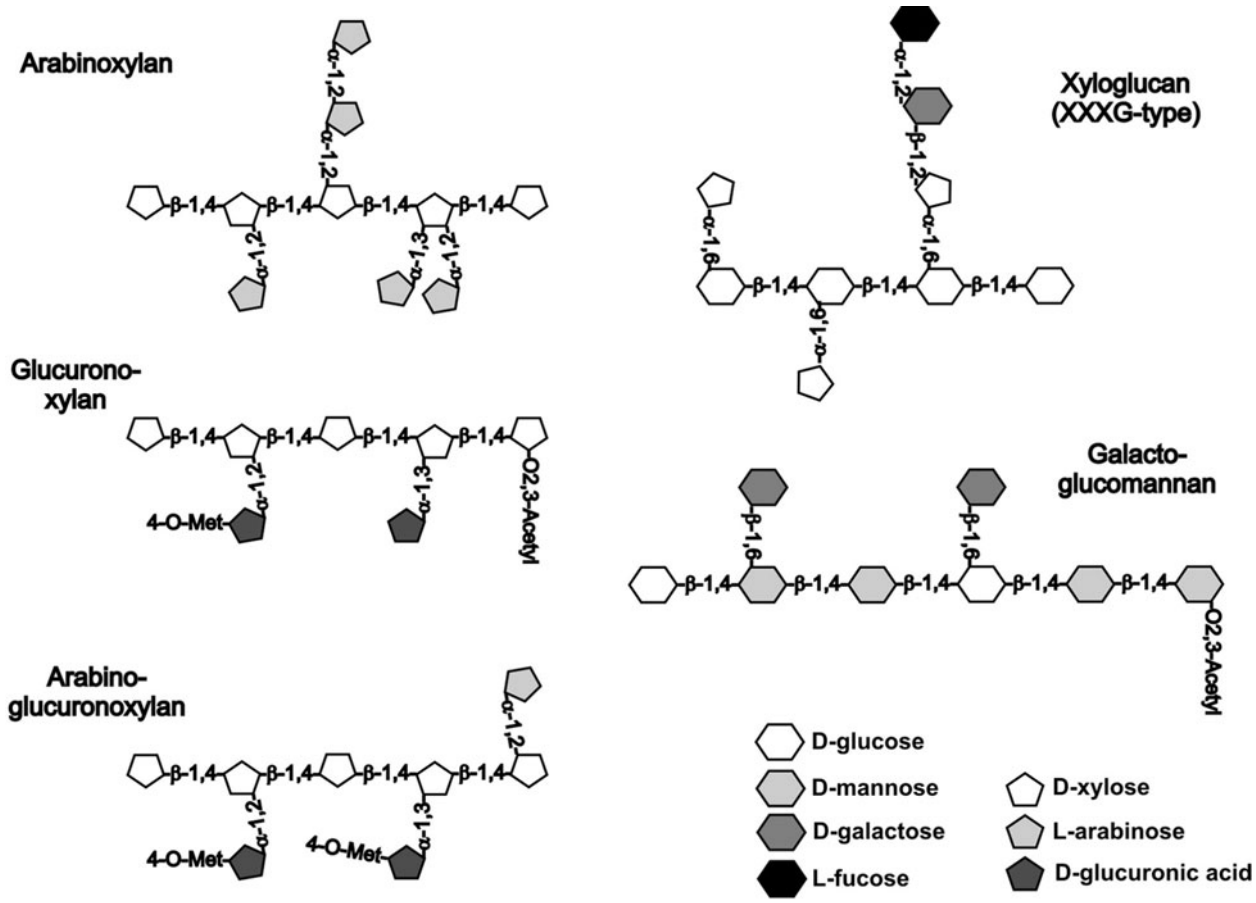

FIGURE 2 Cellulose degradation and regulation. Cellulose is extracellularly degraded by the enzymatic attack of three types of enzymes including cellbiohydrolase (CBH), endoglucanase (EG), and β-glucosidase (BGL). Swollenin (SWO) disrupts the crystalline structure of the cellulose and supports the enzymatic cellulose breakdown. Negative-acting (CRE1, HAP complex, and ACE1) and positive-acting (ACE2 and XYR1) regulators control cellulase expression on the level of transcription.

repressor, ACE1, was described to regulate (hemi)cellulase expression (Aro et al., 2003; Saloheimo et al., 2000). In contrast, in *A. niger* this regulation appears to be solely driven by an interplay between the XYR1 orthologue XlnR and the carbon catabolite repressor CreA. This is supported by the findings that in *A. niger* XlnR binds to single *cis*-acting elements in the upstream regulatory regions of the genes subject to its regulation, whereas in *T. reesei*, only double sites of the respective binding motif have proved to be functional up to now and may be necessary to bind the XYR1/ACE2 complex. The implications of this regulation have been described in detail (Stricker et al., 2008).

The XYR1/XlnR proteins belong to the class of zinc binuclear cluster domain proteins. The binuclear cluster that forms the DNA-binding domain is located at the N terminus of the protein and is almost identical in *T. reesei* and *A. niger*. In addition, XlnR/XYR1 contains another fungal specific transcription factor domain (Pfam domain number PF04082) that is found in many fungal *trans*-acting proteins controlling metabolic processes, although its function is unknown (MacPherson et al., 2006). A putative coiled-coil domain is involved in the nuclear localization of XlnR/XYR1. Finally, removal of a 78-amino-acid region at the C terminus of XlnR leads to an increased expression of the XlnR target genes in the absence of an inducer and under conditions of D-glucose repression (Hasper et al., 2004), suggesting that this region of the protein is responsible for regulation.

In addition to genes encoding xylanases, D-xylose also induces the expression of various other hydrolase-encoding genes in *A. niger*, including genes for the side chain-cleaving enzymes α-glucuronidase (*agu*A), acetylxylan esterase (*axe*A), and feruloyl esterase (*fae*A) (de Vries, 2003; de Vries and Visser, 1999). L-Arabinose and L-arabinitol induce the expression of a set of genes encoding enzymes involved in the degradation of arabinoxylan in *A. niger*, e.g., arabinofuranosidases (*afb*A and *afbB*) and arabinoxylan arabinofuranohydrolase (*axh*A) (de Vries and Visser, 2001). Studies carried out with *A. nidulans* suggest that L-arabitol serves as the intracellular inducer of enzymes involved in the hydrolysis of arabinan-containing hemicelluloses. A mutant strain of *A. nidulans* that accumulates high intracellular levels of L-arabitol produces larger amounts of L-arabinofuranosidase and endoarabinases than the wild-type strain (de Vries et al., 1994). A specific transcriptional activator for arabinases of *A. niger* was first proposed by Flipphi et al. (1994), based on the finding that additional copies of the *abfA* or *abfB* genes lead to a decrease in expression of the other *abf* genes. Subsequently, de Groot et al. (2003) recently reported the isolation of two *ara* mutants of *A. niger* that are defective for the induction of genes encoding arabinolytic enzymes but not for the induction of genes encoding enzymes of the xylanolytic system.

In addition to the compounds mentioned above, many other mono- and disaccharides have been shown to induce expression of a set of hemicellulases. In *T. reesei*, growth on D-galactose only induces expression of the α-galactosidase-encoding genes (*agl1* and *agl2*) and the acetyl xylan esterase-encoding gene (*axe1*) (Margolles-Clark et al., 1996). In *A. niger*, D-galactose and D-mannose are involved in the induction of α-galactosidases and β-mannosidases (Ademark et al., 2001).

The expression of xylanase genes in *A. nidulans* has been shown to be dependent on the ambient pH of the culture medium (MacCabe et al., 1998). The components of pH-dependent regulation are described in chapter 31, in which the transactivator protein PacC occupies a central position. Gielkens et al. (1999) also established the pH-dependent regulation of the *abfB* gene encoding α-L-arabinofuranosidase.

BIODEGRADATION OF PECTINS

Pectins are a complex and heterogeneous group of polysaccharides with a backbone of α-1,4-linked D-galacturonic acid residues. This polyuronic acid has domains with varying side chain lengths, which depending on the architecture of the side chains are called "smooth" or "hairy" regions (Pérez et al., 2000; Ridley et al., 2001). Smooth regions consist of linear chains of α-1,4-linked D-galacturonic acid backbone in which some of the terminal carboxyl groups are methylated or partially O-acetylated at O-2 or O-3 of the galacturonic acid. Hairy regions contain abundant and frequently branched side chains, and three types of galacturonans have been distinguished. (i) RG-I consists of D-galacturonic acid and L-rhamnose in a $[\rightarrow 2)$-α-L-Rha-$(1->4)$-α-D-GalA-$(1\rightarrow]_n$ linkage. Approximately 20 to 80% of the L-rhamnose residues are substituted at O-4 by α-L-arabinofuranosyl and/or β-D-galactopyranosyl residues of varying chain length. These α-1,5-linked arabinan side-chains are further decorated with α-1,3-linked L-arabinose residues. The side chains are terminated by α-L-fucose, (4-*O*-methyl)-β-D-glucuronic acid, ferulic acid, and coumaric acid. (ii) RG-II, which is not structurally related to RG-I, consists of a short backbone of α-1,4-linked D-galacturonic acid with substitutions either at the O-2 or the O-3 position (Vidal et al., 2000) of rare sugars such as D-apiose, L-fucose, L-arabinose, D-galactose, and L-rhamnose. (iii) Xylogalacturonans, which have so far been found only in reproductive plant tissues, are similar to homogalacturonans but contain β-D-xylose at the O-3 position.

Fungi have developed a broad spectrum of pectinolytic enzymes which have been studied in most detail in plant pathogens (*Botrytis cinerea*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum*) and also in *A. niger*, which is an industrial producer of polygalacturonases (PGAs). For plant pathogenic fungi, enzymatic depolymerization of pectin is especially important to weaken the surrounding cell wall of the plant cells and to expose other cell wall polymers to degradation by additional plant cell wall-degrading enzymes. Interestingly, fungi depolymerize pectin by using not only hydrolytic enzymes (PGAs) but also enzymes that cleave polysaccharide chains via a β-elimination mechanism, resulting in the formation of a Δ-4,5-unsaturated bond at the newly formed, nonreducing end. These polygalacturonate lyases are classified as pectin lyases (EC 4.2.2.10), pectate lyases (EC 4.2.2.2), and RG lyases (EC 4.2.2.-).

A sequence analysis showed that the overall amino acid similarity of pectinases is rather low, but nevertheless they share a central core consisting of parallel β-strands forming a large right-handed helix defined as parallel β-helix (Jenkins and Pickersgill, 2001). Although the catalytic mechanism of hydrolases differs from that of lyases, the substrate binding sites are found in a similar position, within a cleft formed on the exterior of the parallel β-helix. This structure facilitates binding to, and cleaving of, the buried pectin polymers in the undamaged cell wall, whereas the parallel β-helix fold confers stability. An exception to this rule is the RG lyase from *Aspergillus aculeatus*, which displays a unique arrangement of three distinct modular domains composed of a domain responsible for RG lyase activity, a fibronectin type III, and a CBM domain (McDonough et al., 2004).

PGAs are the most extensively studied class of pectinases. Endo-PGAs (EC 3.2.1.15) catalyze the hydrolytic cleavage of α-1,4 D-galacturonic bonds within the chain,

and exo-PGAs (galacturan 1,4-α-galacturonidase [EC 3.2.1.67]) cleave from the nonreducing end. Both endo- and exo-PGAs belong to GH family 28 and have similar reaction mechanisms and substrate specificities, but their level of sequence identity is surprisingly low (Biely et al., 1996; Henrissat and Bairoch, 1993; Markovic and Janecek, 2001). A factor that significantly influences the activity of PGAs is the number (and distribution) of methyl and acetyl ester groups, as most endo- and exo-PGAs prefer substrates with a low degree of esterification.

Most fungi produce multiple PGA isozymes with a wide range of enzymatic properties, substrate specificities, and pH optima, which reflects the complexity of the pectin polymer in plant cell walls and the need for enzymes capable of cleaving the HG backbone in a variety of structural contexts. In addition, PGAs can have extended N termini, which were suggested to play a role in their interaction with particular regions of the pectin polymer, thus determining their substrate specificity (Gotesson et al., 2002; Parenicova et al., 2000).

The degree of esterification of pectin also influences the functional classification of lyases. Pectate lyases prefer substrates with a low degree of methyl esterification, which are consequently more acidic, and these enzymes are strictly dependent on Ca^{2+} for catalysis. Pectin lyases, on the other hand, favor highly methyl-esterified substrata and do not require Ca^{2+} ions (Jurnak et al., 1996). A summary of the interplay of the various pectinolytic enzymes in the degradation of pectin, taken from studies with *Aspergillus* spp., is illustrated in Fig. 3.

RG-I is degraded by both hydrolases and lyases. Endorhamnogalacturonases were shown to hydrolyze the α-1,4 glycosidic bonds in saponified hairy regions, thereby producing tetra- and hexamers still substituted with D-galactose. Depending on the monosaccharide cleaved from the nonreducing end of the RG-1, two kinds of exo-RGs were described: rhamnogalacturonan α-D-galactosyluronhydrolase and rhamnogalacturonan α-L-rhamnohydrolase, both of which are members of GH 28. RG lyases act on the Rha-(1→4)-α-D-GalA bond, resulting in the formation of Δ-4,5-unsaturated D-galacturonic acid residues at the nonreducing end. Both RG lyase and RG hydrolase display an increase in catalytic efficiency towards deacetylated RG-I; thus, these enzymes work synergistically with RG acetyl esterase to degrade RG-I.

Different types of substituents are found attached to the pectin: acetyl and methyl esters are found on the pectin backbone, and L-arabinose and D-galactose side chains are found in the hairy regions. The enzymes involved in their release from the pectin backbone are presented in Table 1.

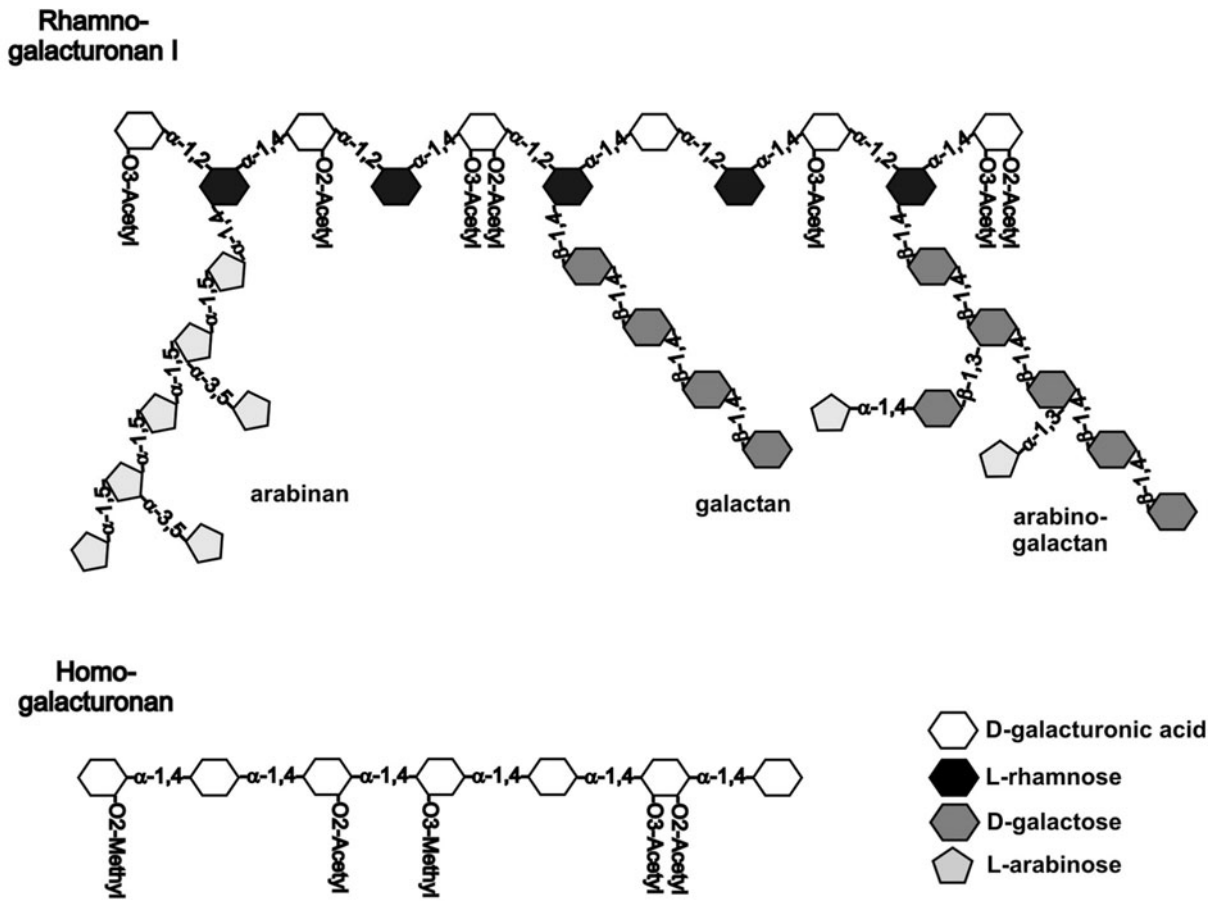

FIGURE 3 Schematic structure of different pectins. Rhamnogalacturonan I and homogalacturonan are the two main structures of the plant cell wall pectin. The main chain of rhamnogalacturonan I (shown on top) is decorated with different arabinan, galactan, and arabinogalactan side chains (hairy region), whereas on the main chain of homogalacturonan, only methyl and acetyl esters are found (smooth region).

In contrast to hemi(cellulase) regulation, little is known about the control of expression of pectinase genes in fungi. Thus far, results have revealed a difference in regulation in saprobic and plant pathogenic fungi. D-Galacturonic acid, polygalacturonate, and sugar beet pectin have been shown to induce expression of nearly all the genes encoding enzymes involved in the degradation of pectin and its side chains in *A. niger* (de Vries and Visser, 2001). It was suggested that all genes encoding pectinolytic enzymes would be under a general pectinolytic regulatory system responding to D-galacturonic acid or a metabolite derived from it (de Vries et al., 2002a, 2002b). In contrast to the regulation of the hemicellulase genes by XlnR/XYR1, the pectinase genes seemed to be controlled by a different regulator. Conserved sites were described for pectinase promoters (Benen et al., 1996), but so far this hypothesis has not yet been tested experimentally, nor has the proposed regulator PecR been identified. Regulation by the carbon catabolite repressor CreA and the pH regulator pacC has been shown in certain cases, but some genes appear to be transcribed constitutively. In *S. sclerotiorum* mutants with loss-of-function alleles in the *A. nidulans pac*C homologue, the expression of an endo-PGA gene important for pathogenesis was shifted to higher ambient pH (Rollins, 2003).

BIODEGRADATION OF LIGNIN

The noncarbohydrate polymer lignin forms an extensive cross-linked network within the cell wall, making up some 15 to 35% of the lignocellulose matrix. It confers structural support to the plant, decreases water permeability, and protects other more easily degradable cell wall components. Lignin consists of three different phenylpropanoid alcohols: coniferyl (guaiacyl propanol), synapyl (syringyl propanol), and *p*-coumaryl (*p*-hydroxyphenylpropanol), which are joined together by C-C and different ether bonds. Softwood contains mainly coniferyl alcohol but no sinapyl alcohol, whereas hardwood consists of equal parts of coniferyl and sinapyl alcohol.

Lignin does not contain glycosidic bonds and consequently cannot be attacked by hydrolases, but its degradation, like that of other aromatic compounds, requires an oxidative attack. White-rot fungi, which belong almost exclusively to the Basidiomycota, are the only group of organisms capable of fully mineralizing lignin. This property is due not only to an efficient enzymatic system for lignin oxidation but also to the fact that these fungi can deal with the low nitrogen content of wood (C-to-N ratio of 500:1) and the presence of toxic and antibiotic compounds.

The degradation of lignin by white-rot fungi can be interpreted as the nonspecific action of peroxidases and laccases to generate free radicals, which then undergo spontaneous cleavage reactions. Peroxidases such as the lignin peroxidases (LiPs) (EC 1.11.1.14), manganese peroxidases (MnPs) (EC 1.11.1.13), and the so-called "versatile peroxidase" (VP) have been described as true ligninases due to their high redox potential, which enables them to oxidize nonphenolic aromatic substrates, which constitute up to 90% of the lignin structure. Peroxidases contain a heme group and require H_2O_2 as a cosubstrate. In the catalytic cycle, LiP becomes oxidized by H_2O_2 (which is thereby reduced to H_2O), and the activated enzyme uses the two electrons to oxidize two substrate units. In this way, veratryl alcohol is oxidized to a short-lived veratryl alcohol cation radical that can either directly oxidize the lignin or react with other more stable carriers that then serve as diffusible mediators of the oxidation. MnPs are closely related to LiPs. They have the same two-electron oxidation catalytic cycle, followed by two subsequent one-electron reductions. MnPs oxidize extracellular Mn^{2+} to Mn^{3+}, which can be chelated by naturally occurring organic acids such as oxalate, fumarate, and malate and can act as diffusible oxidizer on phenolic substrates and oxidize nonphenolic substrates via lipid peroxidation reactions. VP combines the enzymatic properties of LiP and MnP and oxidizes Mn^{2+} and veratryl alcohol. When Mn^{2+} and dimethoxybenzenes are present in the reaction, VP oxidizes hydroquinone in the absence of exogenous H_2O_2 (Martinez et al., 2005).

The crystal structures of LiP and MnP have been solved (Piontek et al., 1993; Poulos et al., 1993; Sundaramoorthy et al., 1994). The prosthetic group (iron protoporphyrin IX) of LiPs is accessible only through a narrow pore (Piontek et al., 2001). The catalytic cycle is common to other peroxidases; however, the position of the iron binding histidine residue in ligninolytic peroxidases is further away from the heme iron. This leads to an increased redox potential. In addition, the specific binding sites for substrate oxidation are unique (Martinez, 2002). MnP differs in its topology of substrate binding: Mn^{2+} oxidation by MnP occurs at a binding site near the cofactor that enables direct electron transfer. In contrast, veratryl alcohol is oxidized by LiP at the surface of the protein by a long-range electron transfer mechanism. This probably reflects the fact that aromatic substrates are unable to enter the LiP/VP proteins (Doyle et al., 1998; Gold et al., 2000; Sundaramoorthy et al., 1997).

Laccases are multicopper phenoloxidases and are generally larger than peroxidases. They perform four one-electron oxidations by reducing O_2 to H_2O and are only able to directly oxidize phenols and aromatic amines by removal of a single electron. They generate phenoxy-free-radical products that can cause polymer cleavage. However, due to their low redox potential, nonphenolic substrates can only be oxidized via the involvement of mediators. Metabolites such as 3-hydroxyanthranilate or some lignin degradation products have been shown to be capable of such mediation mechanisms (ten Have and Teunissen, 2001).

Lignin-degrading fungi require an extracellular source of H_2O_2. Oxidases that are potentially involved in generating H_2O_2 include pyranose 1-oxidase, methanol-oxidase, aryl-alcohol oxidase, and glyoxal oxidase (GLX) (Zhao and Janse, 1996). However, only GLX, a copper radical oxidase with broad substrate specificity for the oxidation of simple aldehydes such as glyoxal and methylglyoxal, appears to be secreted in ligninolytic cultures in liquid medium. GLX also has been implicated in the regulation of peroxidase activity, and it is activated in vitro by lignin peroxidase (Kersten and Cullen, 2007).

Extracellular H_2O_2 may itself also be involved in the generation of highly reactive hydroxyl radicals via the Fenton reaction, as has been described for brown-rot fungi (see above). The involvement of this type of chemical reactions in lignocellulose degradation by white-rot fungi has not yet been investigated.

CDH oxidizes soluble cello- and mannodextrine and uses a wide spectrum of electron acceptors (Fe^{3+}, Cu^{2+}, quinone, and phenoxy radicals). Proposed roles for CDH in the ligninolytic system include (i) the reduction of aromatic radicals formed by ligninolytic enzymes and thus prevention of repolymerization and support of lignin degradation; (ii) the production of ·OH radicals via a Fenton-type reaction to modify cellulose, hemicellulose, and lignin; and (iii) a cooperation with the manganese peroxidases to make

the abundant nonphenolic components of lignin accessible for MnP and laccases. The role of reactive oxygen species in the initial attack of lignin has been described (Hammel et al., 2002) and was reviewed above.

Regulation of the genes encoding ligninolytic enzymes has been studied at the mRNA level mainly in *P. chrysosporium* and has recently been reviewed by Kersten and Cullen (2007). The lignin peroxidases of *P. chrysosporium* are encoded by a minimum of 10 closely related genes, six of which occur in pairs and are transcriptionally convergent, suggesting that portions of this protein family arose by gene duplication events. No apparent correlation was observed between genomic organization and transcript levels. Ligninolytic gene expression is triggered by the depletion of nitrogen, carbon, or sulfur and is regarded as a stress response to nutrient depletion. Expression of the *mnp* gene in *P. chrysosporium* is activated by nitrogen depletion and dependent upon the presence of Mn^{2+} in the culture medium. The presence of putative metal response elements has been reported in some of the *mnp* promoters. Further, *mnp* expression is also induced by H_2O_2, chemical stress compounds, and heat shock. Sequences matching the consensus heat shock element (CN_2GAAN_2 $TTCN_2G$) are found in the promoters of *mnp* genes. It appears that regulation of the *mnp* genes occurs at least at two different levels, since the induction caused by the different stress factors takes place only in nitrogen-limited cultures and the level of induction is in most cases dependent on the presence of Mn^{2+}. Similar to the *mnp* genes, the *lip* genes of *P. chrysosporium* are also regulated differentially in response to carbon or nitrogen starvation.

While conventional laccases are absent from the genome of *P. chrysosporium*, four genes encoding multicopper oxidases (*mco*) were identified and shown to be regulated by Cu^{2+}. In *Saccharomyces cerevisiae*, the Ace1 transcription factor activates the copper-dependent transcription of target genes. An in silico search in *P. chrysosporium* led to the identification of putative ACE elements in the promoter region of *mco1*, one of the four clustered genes encoding multicopper oxidases in *P. chrysosporium*. Addition of Cu^{2+} to cultures increased transcript levels for *mco1* and *mco2* but not *mco4*. In the presence of Cu^{2+}, the *P. chrysosporium* ACE1 induced activation of the promoter of *mco1*, but not that of *mco2* (Canessa et al., 2008).

Expression of laccase-encoding genes is constitutive in many basidiomycetous fungi and can be further enhanced by inducers, such as 2,5-xylidine in *T. versicolor* (Collins et al., 1999), *Trametes villosa* (Yaver et al., 1996), and *A. bisporus* (Smith et al., 1998). It was postulated that constitutively expressed laccases are the first enzymes degrading lignin and that possible degradation products released from lignin by laccases act as inducers that further increase laccase expression and in turn induce the expression of other ligninolytic genes (Scheel et al., 2000).

The expression of the *P. chrysosporium* copper radical oxidase genes is consistent with their role in lignocellulose degradation. Besides the above-mentioned *glx*, six additional copper radical oxidase genes (*cro*) are present in the genome. cDNAs for all six *cro* genes were identified in extensively decayed wood wafers. Transcript patterns determined by competitive reverse transcriptase PCR showed differential regulation among the *cro* genes over a 120-day time course. The absence of *cro4* and *cro6* transcripts at 3 days of colonization suggests that these genes are not essential for hyphal penetration and early decay. Concentrated filtrates of *P. chrysosporium*, grown in defined submerged medium, contained CRO2. In addition to the structural diversity and differential regulation of the *cro* genes, substrate preferences argue also in favor of distinct biological roles, at least for *glx* and *cro2*. The finding that heterologously produced CRO2 oxidizes a glycolaldehyde dimer, but not methylglyoxal (which is the typical substrate for GLX) suggests that the two oxidases are distinguished by catalytic differences (Vanden Wymelenberg et al., 2006).

DEGRADATION OF CHITIN

Chitin is a polysaccharide found in the exoskeleton of crustaceans, such as shrimps and crabs, and insects, and it is the main structural component of the fungal cell walls in the phyla Ascomycota and Basidiomycota. About 10 gigatons of chitin are annually produced in the biosphere. Chitin is composed of β-1,4-linked units of the amino sugar *N*-acetylglucosamine and is the main source for the production of the polymer chitosan, a partially deacetylated form of chitin, and derivatives thereof, which are used in a number of applications, such as flocculating additives in wastewater treatment, wound-healing agents, and additives in dietary products (Tharanathan and Kittur, 2003).

Filamentous fungi have developed a complex chitinolytic enzyme machinery, and their genomes typically contain between 10 and 25 different chitinases. The reasons why fungi have so many chitinases are not well understood. Potential physiological roles of fungal chitinases have recently been reviewed in detail (Seidl, 2008) and include (i) degradation of exogenous chitin present in dead hyphal fragments from other fungi or in the exoskeletons of dead arthropods; (ii) cell wall remodeling during hyphal growth, branching, hyphal fusion, and autolysis; and (iii) competition and defense against other fungi or arthropods in the fungal habitat, including the attack of other fungi (mycoparasitism), insects (entomopathogenic fungi), or nematodes (nematode-trapping fungi).

Many aspects of chitin degradation resemble that of cellulose and have potential impacts on the development of second-generation ("lignocellulosic") bioethanol (Eijsink et al., 2008). Chitinolytic enzymes can be divided into *N*-acetylglucosaminidases and chitinases. *N*-Acetylglucosaminidases (GH 20 [EC 3.2.1.52]) catalyze the hydrolysis of the dimer *N*,*N*′-diacetylchitobiose $(GlcNAc)_2$, and chitinases (GH 18 and 19 [EC 3.2.1.14]) cleave the β-1,4-linkages in chitin chains, resulting in the release of short-chain chito-oligomers. Fungal chitinases belong to GH 18, members of which form oligosaccharide fragments with a β-anomeric conformation (retaining mechanism) (Brameld and Goddard, 1998; Brameld et al., 1998). Traditionally, chitinases were divided into endo- and exochitinases, the former degrading chitin from any point along the polymer chain and forming random-length products, while exochitinases cleave from the nonreducing chain end and the released product is $(GlcNAc)_2$. However, the enzymatic properties of chitinases are more complex and versatile than reflected in the simple "exo-/endo" classification, and a more appropriate way to classify them could therefore be to group them into processive and nonprocessive enzymes (Eijsink et al., 2008). Processive chitinases do not release the substrate after hydrolytic cleavage but instead slide it through the active-site tunnel for the next cleavage step to occur. The presence of a carbohydrate binding domain can enhance processivity but is not essential. In contrast, nonprocessive chitinases dissociate completely from the substrate after hydrolysis. For nonprocessive enzymes

this leads to different substrate degradation patterns with a homogenous distribution of medium chain (6- to 30-mer) products and for processive enzymes to remnants of the polymeric substrate and only 2- to 8-mer short-chain degradation products (Horn et al., 2006a, 2006b).

GH 18 chitinases are subdivided into so-called "chitinase classes III and V," or fungal/plant (class III) and fungal/bacterial (class V) chitinases. This distinction is indeed useful, as it also indicates different enzymatic mechanisms: in class V enzymes, the substrate-binding grooves are deep and tunnel shaped, whereas in class III they are shallow and open. The consequence of these different architectures of the substrate-binding pockets is that class V enzymes show exoacting activities and are processive, whereas class III enzymes are endochitinases and act nonprocessively.

In general, fungi have a high number of GH 18 chitinases in their genomes, with several *Aspergillus*, *Hypocrea/Trichoderma*, and *Fusarium* spp. having between 18 and 32 such chitinases. *N. crassa* and *M. grisea* have fewer, but still possess 12 and 14 chitinases, respectively. A rare exception is *Ustilago maydis* with only two chitinases. In contrast to the number of GH 18 chitinases, only two or three GH 20 *N*-acetylglucosaminidases are present in fungal genomes. This suggests that chitinases rather than *N*-acetylglucosaminidases dictate the substrate depolymerization potential of a fungus and are the key elements for chitin degradation. A phylogenetic analysis of chitinases from different fungal genomes revealed that they can be divided into three different subgroups (Seidl et al., 2005): subgroup A (corresponding to class V enzymes), subgroup B (class III enzymes), and the novel, large subgroup C, which comprised high-molecular-weight chitinases that as yet have not been investigated in any fungus. The catalytic domains of subgroup C chitinases are similar to the processive enzymes from subgroup A, but the overall domain structure of subgroup C is completely different from subgroups A and B. The distribution of GH 18 chitinases among these three subgroups varies widely in different fungi and appears to be correlated with their lifestyle and habitat (Karlsson and Stenlid, 2008). Features of fungal chitinases from subgroups A through C are summarized in Table 2.

The differentiated regulation of individual filamentous fungal chitinases and the synergism that exists between various chitinases are only beginning to be understood. Analysis of the promoter regions and gene expression studies of selected chitinases and *N*-acetylglucosaminidases in *Trichoderma* revealed that these two types of enzymes are induced and regulated by completely different inducers. While chitin is poorly utilized by *T. atroviride*, it induces both chitinases and *N*-acetylglucosaminidases (Mach et al., 1999). On the other hand, *N*-acetylglucosamine, the monomer of chitin, is an excellent carbon source for *T. atroviride* but only induces *N*-acetylglucosaminidases. A number of studies have focused on the induction of chitinases during the parasitic attack of other fungi or insects, and these are reviewed in detail in chapter 42. Transcriptional evidence for the regulation of chitinases by chitin as a carbon source and also by starvation and subsequent autolysis has been obtained in a number of studies involving *T. atroviride*, *A. nidulans*, and *A. fumigatus* (Seidl, 2008). So far the best-studied chitinase is *T. atroviride* CHI18-5 (ECH42; subgroup A), of which orthologues have been cloned and characterized in several fungal species. This chitinase is strongly conserved among the fungal kingdom and is abundantly expressed during growth on chitin and in autolytic growth phases. Its transcription is further regulated by light, carbon catabolite repression, and nitrogen source. Subgroup B chitinases seem to have dual roles. Cell wall-anchored chitinases have recently been shown to be localized at polarized growth sites and are implicated in chitin remodeling during hyphal growth (Yamazaki et al., 2008). whereas small subgroup B chitinases, containing carbohydrate binding domains, are involved in exogenous chitin degradation; for example, *T. atroviride chi18-12* (*chi33*) is regulated by stimuli similar to those of *chi18-5* (de las Mercedes Dana et al., 2001). However, other chitinases such as *T. atroviride chi18-13* (*ech30*; subgroup B) and the only studied chitinase from subgroup C, *T. atroviride chi18-10*, are not induced by growth on colloidal chitin or by starvation but only by growth on fungal cell walls (Seidl et al., 2005). However, the specific inducers responsible have not been identified.

CATABOLISM OF MAJOR NONGLUCOSE CARBOHYDRATES AND THEIR REGULATION

D-Xylose and L-Arabinose Catabolism

The catabolism of the pentoses D-xylose and L-arabinose by fungi is unique, as no isomerization reactions similar to those observed in the bacterial catabolic pathway are involved here. An exception is perhaps anaerobic fungi, because the presence of a bacterial pentose catabolic pathway was shown in *Piromyces* sp. (Harhangi et al., 2003). A phylogenetic analysis of its xylose isomerase shows that it clusters with plant enzymes and enzymes from bacteria with a low G+C content in their DNA. However, the "fungal" pathways have been detected in all other fungi tested so far, notably in *Penicillium* spp., *Aspergillus* spp., and *T. reesei*. Their reaction schemes are given in Fig. 4 and demonstrate that the two degradative pathways are interconnected: D-xylose is first reduced to xylitol, which is then reoxidized to D-xylulose and phosphorylated to D-xylulose-

TABLE 2 Properties of fungal chitinases from phylogenetic subgroups A, B, and C

Subgroup	Molecular mass (kDa)	Localization	Catalytic domain architecture	CBMs	No. present in filamentous fungi
A	40–50	Mostly extracellular; some intracellular and in ER	Processive	—[a]	4–6
B	I: 90	I: Cell wall anchored (GPI)	Nonprocessive	I: —	I: 1–2
	II: 30–45	II: Extracellular		II: Can contain CBM 1, CBM 19, CBM 5/12	II: 0–5
C	100–150	Extracellular	Processive	Multiple motifs; CBM 18, LysM	Ascomycota, 3–12; Basidiomycota, 0

[a]—, no data available.

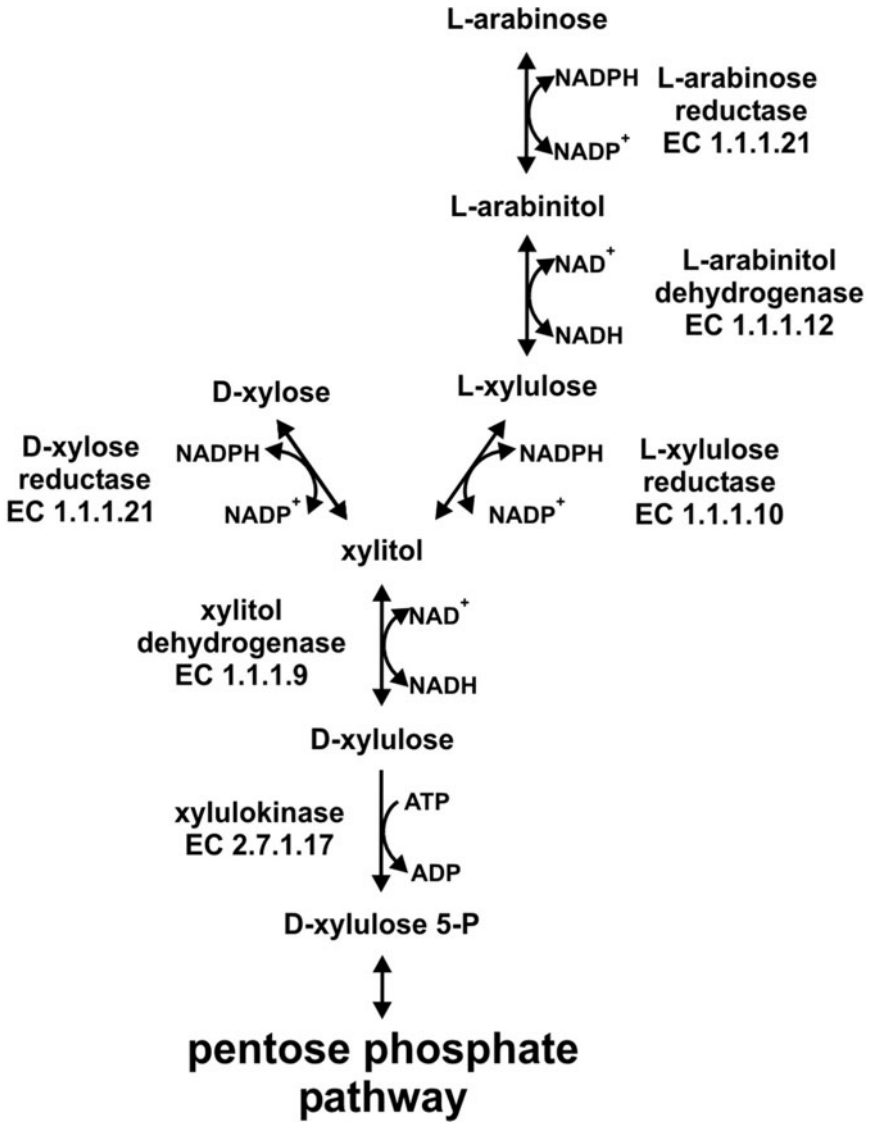

FIGURE 4 Fungal D-xylose and L-arabinose catabolism. The fungal catabolic pathway for D-xylose and L-arabinose is an interconnected pathway that channels both sugars in the pentose phosphate pathway. NADPH-dependent reductions alternate with NAD^+-dependent oxidations before D-xylulose is finally phosphorylated by xylulokinase to D-xylulose 5-phosphate. In *T. reesei* the main enzyme for the first step in both pathways is the D-xylose reductase XYL1.

5-phosphate, a metabolite of the pentose phosphate pathway. L-Arabinose is catabolized in a similar way, by being first reduced to L-arabinitol and then oxidized to L-xylulose. L-Xylulose is then reduced to xylitol, which follows the same path as described above for D-xylose catabolism. Genes encoding the enzymes for this pathway are present in all fungal genomes, in some cases as multiple copies, whereas pentose isomerase genes were not detected. The nature of the enzymes catalyzing the first reduction of D-xylose and L-arabinose, respectively, seems to be different in *A. niger* and *T. reesei*; in the former, an enzyme with preferred specificity for L-arabinose has been described, whereas in *T. reesei* a single aldose reductase accounts for most of the activity on both D-xylose and L-arabinose. Interestingly, two L-arabinitol dehydrogenases and even four L-xylulose reductases are present in *A. niger*, indicating a strong amplification of this pathway and/or specialization of the respective multiple enzymes in the catabolism of different pentoses and D-galactose (see below). The multiplicity could contribute to a proper balance between induction of hemicellulolytic enzymes and the subsequent catabolism of the resulting pentose monomers.

Intriguingly, several fungi also contain a copy of a putative phosphoketolase (EC 4.1.2.9) gene. This raises the possibility for a shortcut catabolism of D-xylulose-5-phosphate to glyceraldehyde-3-phosphate and acetyl-phosphate, bypassing the pentose phosphate pathway (Flipphi et al., 2009). Further, a gene encoding an NADP(+)-dependent D-xylose dehydrogenase was identified in *T. reesei* (Berghall et al., 2007; Hilditch et al., 2007). The enzyme had highest activity with D-xylose and significantly lower activities with other aldose sugars. The role of this enzyme is unclear, however, because the fungus is unable to grow on D-xylonic acid.

The transcriptional activator XlnR/XYR1, which is responsible for cellulase and xylanase regulation in *T. reesei* and *Aspergillus* species such as *A. niger*, was also shown to be necessary for D-xylose reductase induction (Hasper et al., 2000; Stricker et al., 2006) during growth on D-xylose. An interesting difference between the two fungi is that *A. niger* has a second D-xylose-reducing enzyme, which enables reasonable growth on D-xylose, while a *T. reesei* deletion strain is almost unable to grow on D-xylose. The reductase responsible for the remaining D-xylose reductase activity in *A. niger* is probably the L-arabinose reductase, which shows overlapping substrate specificity with the D-xylose reductase.

D-Galactose Catabolism

The predominant pathway for D-galactose catabolism in most eukaryotes is the Leloir pathway (Fig. 5) (Frey, 1996; Holden et al., 2003).Three enzymes are necessary to convert D-galactose to D-glucose-1-phosphate before phosphoglucomutase finally converts it to glycolytic pathway intermediate D-glucose-6-phosphate: galactokinase (Gal1), D-galactose-1-phosphate uridylyltransferase (Gal7), and UDP-galactose 4-epimerase (Gal10). A physiologically important feature of galactokinases in general is that they are strictly specific for α-D-galactose, which is produced by the aldose 1-epimerase (D-galactose mutarotase) from the β-anomer. In most yeasts (*S. cerevisiae* and *Kluyveromyces lactis*) both epimerase domains are found in the bifunctional Gal10, while in prokaryotes the aldose 1-epimerase is encoded by a separate gene.

The genomes of all fungi contain orthologues of *S. cerevisiae GAL1*, *GAL7*, and *GAL10*. A detailed characterization of them has been performed for *T. reesei* (Seiboth et al., 2004; Seiboth et al., 2002a, 2002b): GAL1 and GAL7 are conserved proteins, whereas the GAL10 of *H. jecorina* and other mycelial fungi consists only of a UDP-galactose 4-epimerase domain and lacks the C-terminal aldose 1-epimerase domain. Also the genomic organization of the Leloir pathway genes in filamentous fungi reveals a major difference in comparison to yeasts. In the latter, they are organized in a cluster, whereas they are dispersed throughout

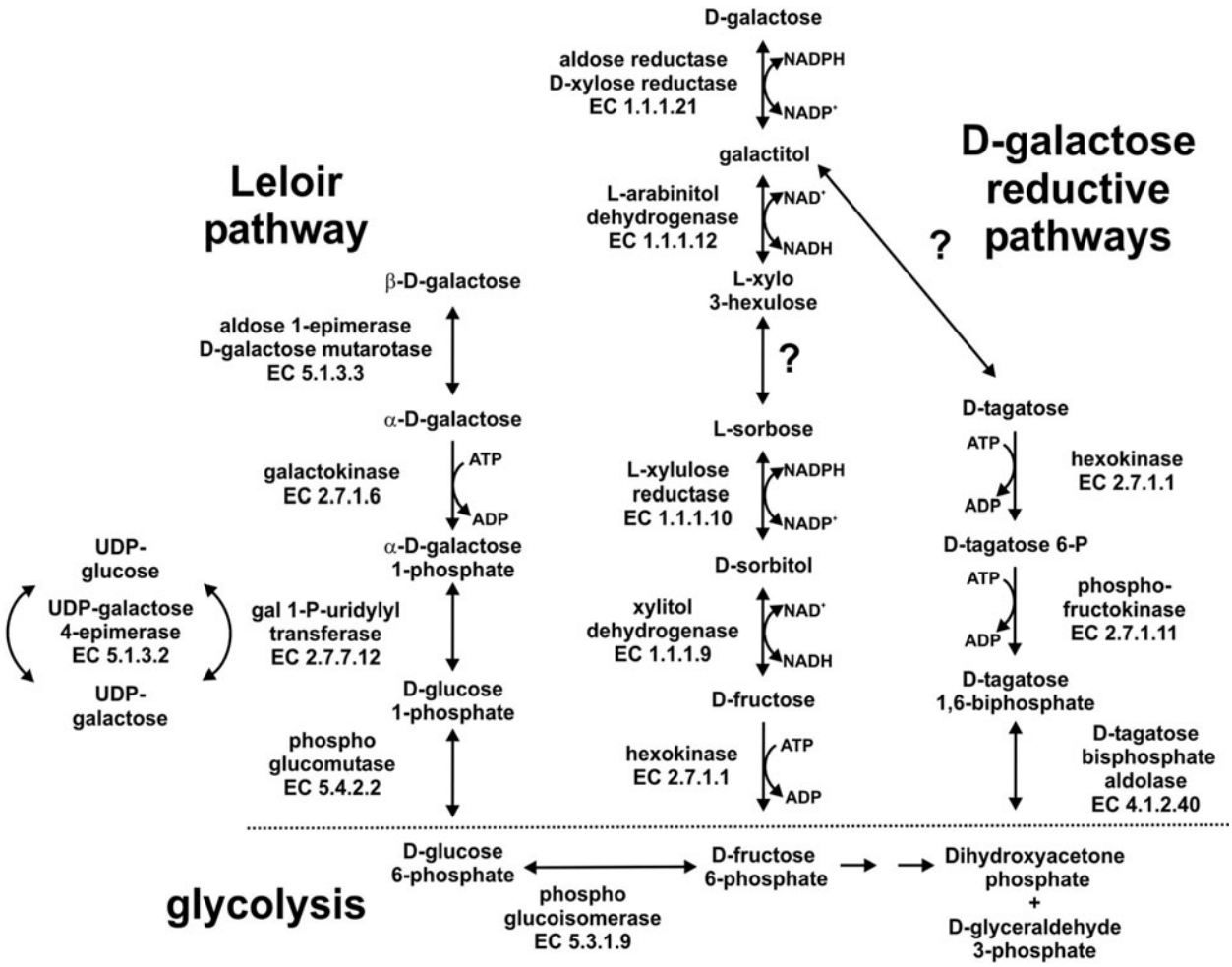

FIGURE 5 Fungal D-galactose catabolism. D-Galactose occurs in nature in the two anomeric forms α- and β-D-galactose. The galactokinase of the classical Leloir pathway (left) is specific for α-D-galactose, and therefore, β-D-galactose has to be epimerized to the α-anomer before it can enter this pathway. A second pathway (right) was found recently in *T. reesei* and *A. nidulans*. It starts with the reduction of both anomeric forms of D-galactose to galactitol. Two hypothetical drafts for the further degradation of galactitol are summarized.

the genome in mycelial fungi. A synteny of their genomic organization in different filamentous fungi suggests that the *gal* genes diverged between yeast and multicellular fungi early during evolution.

Filamentous fungi are able to catabolize D-galactose via other pathways in addition to GAL1/GAL7/GAL10. Similar to what is observed with *T. reesei*, activities of the Leloir pathway enzymes are also found in *A. nidulans* under noninducing conditions and these enzyme activities are induced by D-galactose (Roberts, 1963, 1970). But mutants lacking galactokinase or D-galactose-1-phosphate uridylyltransferase activity were still able to grow on D-galactose at a pH of >7.0. Roberts speculated that the pathway found at pH values of >7 starts with an oxidative step by a D-galactose oxidase. This would be consistent with the presence of genes encoding a galactose oxidase (EC 1.1.3.9), a galactolactonase (EC 3.1.1.25), and a galactonate dehydratase D-galactonate hydrolyase (2-dehydro-3-deoxy-D-galactonate-forming [EC 4.2.1.6]) in all *Aspergillus* genomes. The relevance of this pathway for catabolism of D-galactose is unclear, however, as the L-galactonate dehydratase gene of *T. reesei* has been shown to be mainly involved in the degradation of pectin-derived D-galacturonate (Kuorelahti et al., 2006).

In *T. reesei* and *A. nidulans*, there is now evidence for a reductive D-galactose catabolic pathway. This pathway makes use of reactions and enzymes of L-arabinose/D-xylose reductase catabolism and in analogy to these pathways ends with a phosphorylation step of D-fructose (Fig. 5). The enzyme initiating this pathway is the same as that for D-xylose and L-arabinose catabolism, i.e., D-xylose reductase XYL1 (Seiboth et al., 2007), because Δ*xyl1* strains show not only a strongly reduced growth on the two pentoses D-xylose and L-arabinose but also a decreased growth on D-galactose, and consequently, Δ*gal1*Δ*xyl1* strains are almost unable to grow on D-galactose. It was therefore concluded that this pathway is necessary to handle the β-D-galactose moiety of β-linked D-galactose side chain in hemicelluloses, because aldose reductases—in contrast to galactokinase—are able to reduce both anomers of D-galactose and their presence thus makes *T. reesei* independent from the aldose 1-epimerase step of the Leloir pathway. This hypothesis is further supported by the fact that the reductive D-galactose-degrading pathway is induced in the presence of β-linked D-galactose-containing saccharides such as lactose. Recently, it was established that no aldose 1-epimerase activity is produced during growth on lactose or D-galactose (Fekete et al., 2008). There is uncertainty in this pathway regarding the product of the galactitol dehydrogenase reaction. While the logical candidate would be D-tagatose, Fekete et al. (2004) observed the accumulation of L-sorbose in vivo, whereas the purified enzyme from *T. reesei* (Pail et al., 2004) and *A. nidulans* produces L-xylo-3-hexulose from galactitol. This step therefore needs further clarification.

Fungal genome annotations offer still another pathway for D-galactose utilization: fungal genomes contain several class II D-tagatose bisphosphate aldolases (EC 4.1.2.40) (Flipphi et al., 2009). While the class II type A aldolases are highly specific for fructose-bisphosphate, the smaller class II type B proteins (such as the tagatose-bisphosphate aldolases) would have a wider substrate range (Hall et al., 2002). The *Aspergillus* class II type B proteins show similarity to two *Escherichia coli* proteins involved in the degradation of galactitol and *N*-acetylgalactosamine, encoded by *gatY* and *kbaY* (Nobelmann and Lengeler, 1995; Reizer et al., 1996). Such an enzyme could thus split D-tagatose-1,6-bisphosphate (and possibly also other keto-hexose bisphosphates) into dihydroxyacetone-phosphate and D-glyceraldehyde-3-phosphate. In this hypothetical pathway fungi may be able to degrade D-galactose by phosphorylating the ketohexose arising from galactitol either directly or after epimerization to D-tagatose, followed by subsequent cleavage into the two glycolytic triose-phosphates. We note that such a scenario is not in conflict with the genetic analyses done with *A. nidulans* and *T. reesei*. A summary of the catabolic pathways for D-galactose is given in Fig. 5.

In contrast to the *GAL* regulon in *S. cerevisiae*, which represents a model system for eukaryotic gene regulation, less is known about the factors that regulate both D-galactose catabolizing pathways. A major difference between yeast and filamentous fungi with respect to the regulation of the Leloir pathway genes occurs at the level of transcription: in *S. cerevisiae*, the induction of the *GAL* genes is controlled by the interplay of three proteins, i.e., a transcriptional activator, Gal4, a repressor, Gal80, and an inducer, Gal3. Induction appears to occur as a result of a D-galactose- and ATP-dependent interaction between Gal3 and Gal80, which results in the formation of a transcriptionally active Gal4-Gal80-Gal3 complex (Bhat and Murthy, 2001). Gal3 shares a high degree of sequence identity to the galactokinase Gal1 but has no galactokinase activity due to the lack of the amino acid doublet SA in the ATP binding domain (Platt et al., 2000). In contrast, in *T. reesei* all three genes are formed constitutively, and *gal1* and *gal7*, but not *gal10*, can be further induced (about two- to threefold) by D-galactose. This difference is further highlighted by the absence of orthologues of *GAL3* and *GAL4* in the fungal genomes of *T. reesei* and *A. nidulans*. Hartl et al. (2007) demonstrated that in *T. reesei*, in contrast to the two yeasts *S. cerevisiae* and *K. lactis*, neither the galactokinase activity nor the galactokinase protein is needed for the constitutive or induced level of *gal7* transcription. These data document an important difference in D-galactose metabolism between yeasts and filamentous fungi and show that the latter developed a fundamentally different mechanism for D-galactose induction. These differences may originate from the abundance of D-galactose in the plant polysaccharides available in the natural habitats of filamentous fungi and the role of D-galactose as an important component of the fungal cell walls and glycoproteins.

In the alternative, reductive pathway, induction of the XYL1 aldose reductase catalyzing the first step from D-galactose to galactitol is known to require the XlnR/XYR1 regulator during growth on D-galactose and lactose, but the induction could be fully recovered by galactitol (Seiboth et al., 2007).

D-Galacturonate Catabolism

D-Galacturonate occurs mainly in pectin, and the fungal pathway for its degradation has only recently been investigated in *T. reesei*; D-galacturonate is first reduced to L-galactonate by an NADPH-coupled D-galacturonate reductase GAR1 (Kuorelahti et al., 2005; Liepins et al., 2006). In the second step, L-galactonate is converted to L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate) by a dehydratase LGD1 (Kuorelahti et al., 2006). L-Threo-3-deoxy-hexulosonate is then cleaved by an aldolase to pyruvate and L-glyceraldehyde. The L-glyceraldehyde can then be converted to glycerol (Liepins et al., 2006), most likely by the GLD1 glycerol dehydrogenase. Although the role of GLD2 is to facilitate glycerol formation under osmotic stress conditions, the authors hypothesized that GLD1 is active in D-galacturonate catabolism because of its expression under these conditions.

N-Acetylglucosamine Catabolism

The degradation of chitin ultimately results in the formation and uptake of *N*-acetylglucosamine (GlcNAc). Its catabolism has not yet been studied in filamentous fungi but is well known from the human pathogenic yeast *Candida albicans*, where it is an important aspect of its pathogenicity (Kumar et al., 2000). The catabolic pathway consists of hexokinase, which phosphorylates GlcNAc, followed by a GlcNAc-6-phosphate deacetylase, yielding glucosamine-6-phosphate, and the glucosamine-6-phosphate deaminase. The product of the last reaction is fructose-6-phosphate, which is further catabolized via the glycolytic pathway. The genes of GlcNAc catabolism exist in a cluster in the genome of *C. albicans* and are also clustered in filamentous fungi. All three genes are temporally and coordinately induced by GlcNAc, suggesting a common regulatory mechanism for them. Two regions were revealed to be responsible for GlcNAc induction: 5′-GGAGCAAAAAAATGT 3′ (box A) and 5′-ACGGTGAGTTG 3′ (box B) are recognized and bound by at least two inducible activator proteins directing the regulation of gene expression.

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PHOTOBIOLOGY AND CIRCADIAN RHYTHMS

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Cellular and Molecular Biology of Filamentous Fungi
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Light Sensing

LUIS M. CORROCHANO AND JAVIER AVALOS

Fungi sense and interact with the surrounding environment by changing their pattern of growth and their metabolic activities (Bahn et al., 2007), and light is one of the environmental signals that regulate many aspects of fungal biology. A prominent example of the effect of light in fungi is the regulation by light of fungal development and behavior (Corrochano and Galland, 2006). Blue light is the type of light most associated with fungal photomorphogenesis, but other wavelengths can be effective, as shown by the activation of conidiation by red light in *Aspergillus nidulans* (Mooney and Yager, 1990) and by near-UV light in *Alternaria tomato* (Kumagai, 1989). The effect of blue light is often either stimulatory or inhibitory to a developmental transition. In addition, the near-UV induction of conidiation in *A. tomato* can be inhibited by exposure to blue light (Kumagai, 1989), and blue and red light waves are required for maximum conidial production in *Aspergillus* (Purschwitz et al., 2008), suggesting the operation of complex photoreceptor systems. Blue light can activate fungal metabolic pathways or direct the growth of fungal structures, as shown by the activation by light of carotene biosynthesis in *Phycomyces blakesleeanus* and *Neurospora crassa* and the phototropism of the fruiting body of *Phycomyces* (Cerdá-Olmedo, 2001; Linden et al., 1997a).

Photoreceptors are molecules that receive photons through specialized light-absorbing chromophores and transduce the photon energy into the cell to promote a response. Photoreceptors are key elements in the light transduction pathway, since the basic properties of a photoresponse, such as the color of light (energy) that can be perceived or the sensitivity (threshold) of the photoresponse, depend on the photoreceptor that mediates a particular photoresponse. Photoreceptor molecules have been described in all types of living organisms (Briggs and Spudich, 2005), including fungi (Corrochano, 2007; Herrera-Estrella and Horwitz, 2007; Idnurm and Heitman, 2005b; Purschwitz et al., 2006). The isolation and characterization of fungal photoreceptors were initiated by the isolation of the *Neurospora wc-1* and *wc-2* genes, required for all the photoresponses in this fungus, followed by the characterization of the *wc-1* gene product, WC-1, as a photoreceptor with DNA-binding capacity that operates with its partner, WC-2. The identification of genes with similarities to *wc-1* and *wc-2* in ascomycete, basidiomycete, and zygomycete fungi, many of them also required for fungal photoresponses, led to the proposal that the WC complex arose early in fungal evolution to regulate fungal photoresponses as a photoreceptor and transcription factor (Idnurm and Heitman, 2005a). In addition, the completion of several fungal genomes has allowed the identification of additional fungal photoreceptor genes, many of them unexpected (Borkovich et al., 2004; Galagan et al., 2003). In this chapter we summarize our current knowledge of the mechanism of light sensing in fungi, including a description of fungal photoreceptors and their mechanism of action, and describe the fungal responses that are mediated by these photoreceptors.

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FUNGAL PHOTORECEPTORS

LOV-Domain Photoreceptors: the *Neurospora* WC Complex

The molecular mechanisms of fungal photoreception have been investigated in most detail in the ascomycete *N. crassa*. Blue-light responses in *Neurospora* include the induction of sporulation, sexual development, synthesis of mycelial carotenoids, and the regulation of the circadian clock; all of these responses require the products of the *wc-1* and *wc-2* genes (Linden et al., 1997a).

WC-1 is a protein with a Zn finger, two PAS domains for protein-protein interactions, a putative transcriptional activation domain, a nuclear localization signal, and a chromophore-binding domain (Ballario et al., 1996) (Fig. 1). The chromophore-binding domain binds the flavin chromophore FAD, allowing WC-1 to act as a photoreceptor (Froehlich et al., 2002; He et al., 2002). The WC-1 flavin-binding domain (named LOV) has been described in other photoreceptor proteins, most notably in plant phototropins (Christie, 2007). The primary photochemical event in

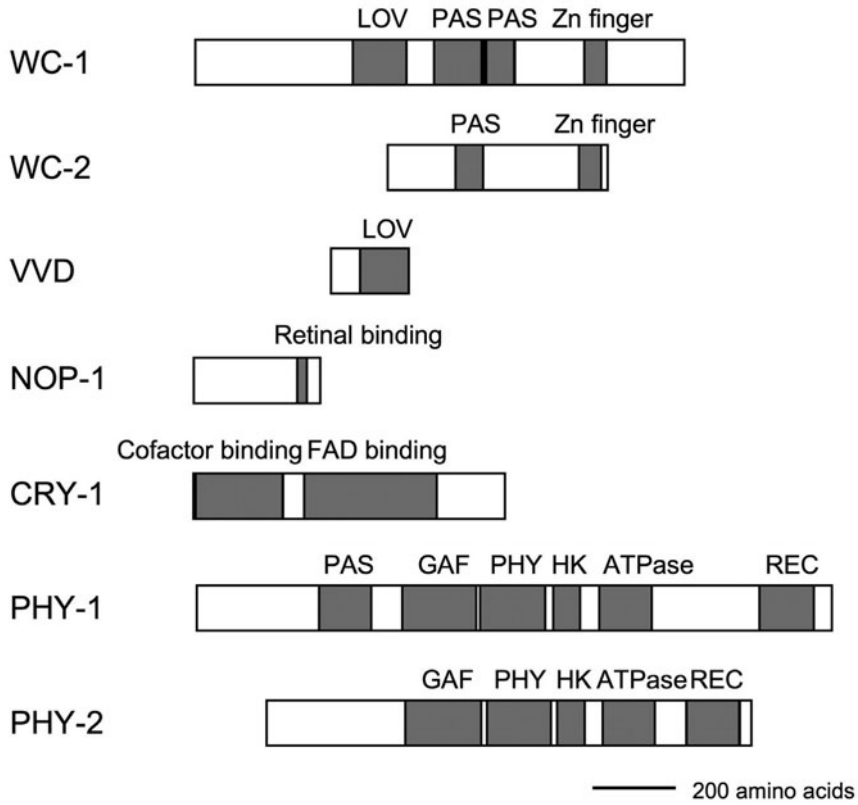

FIGURE 1 Photoreceptor proteins in *Neurospora crassa*. Shown are the LOV-domain photoreceptors WC-1 and VIVID (VVD) together with WC-2, the protein that interacts with WC-1 to form the photoresponsive WC complex. Other photoreceptors identified in the *Neurospora* genome are a rhodopsin (NOP-1), a cryptochrome (CRY), and two phytochromes (PHY-1 and PHY-2). LOV-domain photoreceptors contain the flavin chromophore-binding domain (LOV) and may also contain the protein-interaction domains (PAS), and the Zn finger domain. Rhodopsins contain the retinal-binding domain. Cryptochromes contain the FAD chromophore-binding domain and the domain for binding the antenna cofactor. Phytochromes contain an amino-terminal sensory domain and a carboxy-terminal output domain. The sensory domain involved in binding the bilin chromophore is composed of three domains (PAS, GAF, and PHY). The output domain is composed of the histidine kinase domain (HK), the ATPase domain, and the response regulator domain (REC), which is likely involved in relaying the light signal to other proteins. The identity and the position of the domains were predicted using the SMART database (http://smart.embl-heidelberg.de/) (Letunic et al., 2006) and the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer and Bryant, 2004).

phototropins is the formation of a flavin-cysteinyl adduct at a cysteine of the LOV domain (Salomon et al., 2000), suggesting that WC-1 activation occurs through the formation of a light-dependent flavin-cysteinyl adduct. This mechanism is further supported by the lack of light-dependent gene activation when the conserved cysteine of the WC-1 LOV domain is replaced by serine (Cheng et al., 2003a).

WC-2 is a protein with a Zn finger, a single PAS domain, a putative transcriptional activation domain, and a nuclear localization signal (Linden and Macino, 1997) (Fig. 1). WC-1 and WC-2 interact through their PAS domains to form a WC complex (Ballario et al., 1998; Cheng et al., 2001b, 2002, 2003b; Denault et al., 2001; Talora et al., 1999) that binds the promoter of light-inducible genes (Ballario et al., 1996; Belden et al., 2007; Froehlich et al., 2002, 2003; He and Liu, 2005; Linden and Macino, 1997; Schafmeier et al., 2005).

The WC proteins are present in the dark (Denault et al., 2001; Schwerdtfeger and Linden, 2000; Talora et al., 1999) and are preferentially located in the nucleus, although the more abundant WC-2 is also observed in the cytoplasm (Cheng et al., 2001a; Denault et al., 2001; Schafmeier et al., 2005; Schwerdtfeger and Linden, 2000). Nuclear localization of either WC-1 or WC-2 is not affected by light and is not altered by mutations in *wc-2* or *wc-1*, respectively, indicating that nuclear localization does not require a complete WC complex (Schwerdtfeger and Linden, 2000). In the WC complex, WC-1 is the limiting factor while WC-2 is in excess (Cheng et al., 2001b; Denault et al., 2001). However, overexpression of *wc-1* is not sufficient to activate most light-inducible genes (Lewis et al., 2002).

The *wc-1* gene has a complex promoter with three different transcription initiation sites, one of them located within the open reading frame (ORF) itself (Káldi et al., 2006). The *wc-1* gene is induced by light, but *wc-2* is equally expressed in dark- and light-grown mycelia (Ballario et al., 1996; Linden and Macino, 1997). In addition, the transcription of *wc-2* is negatively regulated by WC-1 (Cheng et al., 2003b) through a putative repressor (Neiss et al., 2008). The sequence

responsible for the activation by light of *wc-1* is confined within a DNA segment of 1,179 bp upstream of the ORF (Káldi et al., 2006). The *wc-2* promoter, on the contrary, required only 600 bp upstream of the ORF (Neiss et al., 2008). The structure and function of the WC proteins have been reviewed (Dunlap and Loros, 2005; Liu et al., 2003).

Regulation and Activity of the WC Complex in *Neurospora*: Control of Gene Transcription by Light

The functional domains in WC-1 and WC-2 suggested that these proteins operated as light-dependent transcription factors and that the light transduction pathway in *Neurospora* could be reduced to a handful of elements. A simplified model for light transduction begins with the activation of the WC complex by light, which then activates the transcription of a set of light-regulated genes, allowing the light-dependent accumulation of certain proteins required for light responses. However, an interesting feature of light-dependent gene transcription is that the response to light is transient. Transcription of light-responding genes in *Neurospora* ceases after the illumination time has been extended, and further incubation in the dark is required before they are again transcribed in response to light (Arpaia et al., 1999; Lauter and Yanofsky, 1993; Schwerdtfeger and Linden, 2001, 2003). This feature, named "transcriptional adaptation to light" or "photoadaptation," might allow a quick transcriptional response after environmental changes in light intensities, such as those observed at dawn or sunset, or to allow the precise orientation of reproductive organs towards a source of bright light in a shaded environment. The lack of transcriptional response during adaptation to light can be reversed by increasing the light intensity (Arpaia et al., 1999; Schwerdtfeger and Linden, 2001, 2003), suggesting that photoadaptation is probably a consequence of photoreceptor desensitization. Photoadaptation is modified by mutations in the gene *vivid* (Heintzen et al., 2001; Schwerdtfeger and Linden, 2001, 2003; Shrode et al., 2001) and by inhibitors or mutations of the protein kinase C (Arpaia et al., 1999; Franchi et al., 2005) and requires protein synthesis (Schwerdtfeger and Linden, 2001). A genetic selection system using a *Neurospora* strain carrying the gene for resistance to hygromycin under the control of the regulatory sequence of the light-regulated gene *con-10* allowed the identification of novel mutants altered in gene photoadaptation, but none of the relevant genes have yet been characterized (Navarro-Sampedro et al., 2008). Any model for light-dependent gene regulation by the WC complex must take photoadaptation into account. A detailed analysis of light-dependent transcription using genomic microarrays has identified 5.6% of transcripts showing accumulation after short or long light exposures (early or late genes) (Chen et al., 2009), a number not too different from that obtained with a smaller sample of cDNAs (Lewis et al., 2002). Interestingly, the whole-genome microarray experiments identified several light-responsive genes for transcription factors, and one of them, the GATA family transcription factor SUB-1, was required for the activation by light of late-responsive genes (Chen et al., 2009). These results suggest that the WC complex activates the transcription of *sub-1* so that SUB-1 can activate late-responding genes. The observation of WC complex binding to the promoter of *sub-1* supports this proposal and suggests a signaling cascade of transcriptional regulators in *Neurospora* (Chen et al., 2009).

In order to understand the molecular mechanism of light-dependent gene regulation, most research has focused on the behavior and activity of the WC complex during and after exposure to light. Light causes a decrease in the mobility of the WC complex bound to the promoter, suggesting light-dependent aggregation of WC complexes (Froehlich et al., 2002; He and Liu, 2005). In vivo, the WC complex only binds transiently to the promoter of light-activated genes, in agreement with the observation of transient gene photoactivation (He and Liu, 2005). After activation, the WC complex is predicted to return to the dark level, completing the photocycle. The WC complex has a slow photocycle in vivo, as shown by the observation that after an initial illumination it is necessary to incubate the fungus in the dark for more than 1 h to obtain further gene photoactivation (Arpaia et al., 1999; He and Liu, 2005; Schwerdtfeger and Linden, 2001).

A *Neurospora wc-1* strain lacking the Zn-finger domain is still capable of gene photoactivation (Cheng et al., 2003b), and strains carrying *wc-2* alleles with mutations that change conserved amino acids of the Zn finger domain can photoactivate the *frq* gene (Collett et al., 2002), indicating that the lack of a Zn finger in one of the WC proteins does not completely hinder gene photoactivation. In fact, mutations in some amino acids of the LOV domain allow the photoactivation of *frq* while preventing photoactivation of *al-3*, an indication that genes may differ in their WC-1 requirements for photoactivation (Cheng et al., 2003a).

An additional feature of the response to light is the modification of chromatin, as demonstrated by the observation of a transient light-dependent acetylation of lysine 14 in histone H3 associated with the promoter of the light-inducible gene *al-3*. The relevance of this result was supported by the blind phenotypes observed in a strain with a replacement of lysine 14 by glutamine in histone H3 and in a strain with mutations in the gene *ngf-1* for a histone acetylase (Grimaldi et al., 2006). These findings suggest that light reception by the WC complex promotes a chromatin modification that may allow RNA polymerase better accessibility at light-inducible promoters (Grimaldi et al., 2006).

Light promotes the transcription of *wc-1* (Ballario et al., 1996; Káldi et al., 2006), and phosphorylation of WC-1 (He and Liu, 2005; Schwerdtfeger and Linden, 2000, 2001; Talora et al., 1999). The light-dependent phosphorylation of WC-1 is transient, does not require the presence of WC-2, and leads to WC-1 degradation and its replacement in the WC complex by newly synthesized but inactive WC-1 protein. The photoactivation of *wc-1* requires an active WC complex (Ballario et al., 1996) allowing the light-dependent synthesis of new WC-1 to replace the phosphorylated WC-1 when it is excluded from the WC complex and degraded. In addition, the light-dependent phosphorylation of the WC complex reduces its capacity to bind light-inducible promoters, suggesting that phosphorylation plays a prominent role in the transient activity of the WC complex (He and Liu, 2005; Schafmeier et al., 2005). The amount of WC-1 and the kinetics of the light-dependent phosphorylation are altered by the presence of a mutant form of WC-2, suggesting that WC-2 is necessary to sustain the transience and magnitude of WC-1 phosphorylation (Schwerdtfeger and Linden, 2000; Talora et al., 1999). Some phosphorylation of WC-1 is also observed in the dark. Five phosphorylation sites located downstream of the Zn finger domain are not necessary for the light-dependent phosphorylation but are responsible for alterations in the circadian rhythm of conidiation (He et al., 2005). Protein kinase C (PKC) interacts with WC-1 in vivo and phosphorylates the Zn finger domain in vitro. However, this interaction is only observed in dark-grown mycelia or after 2 h of illumination, when gene photoactivation has ceased (Franchi et al., 2005).

Regulatory mutations in PKC result in changes in the amount of WC-1, with the corresponding changes in gene photoactivation without altering the light-dependent phosphorylation pattern of WC-1, confirming that PKC is a negative regulator of WC-1 (Franchi et al., 2005). These results have suggested that other kinase(s) must be responsible for the observed light-dependent phosphorylation of WC-1.

WC-2 is also phosphorylated after light exposure, but the modification is more subtle, requires the presence of WC-1, does not change with longer light exposures, and is not observed in cytoplasmic WC-2 (Schwerdtfeger and Linden, 2000). The light-dependent phosphorylation was not observed in strains with a mutation in the chromophore-binding domain of *wc-1* or a mutation in the Zn finger domain of *wc-2* (Schwerdtfeger and Linden, 2000).

Abundance, phosphorylation, and activity of the WC complex are also controlled by FRQ, a master regulator of the *Neurospora* circadian clock. The oscillation in the amount of FRQ during a circadian cycle results in changes in the abundance, phosphorylation, and activity of the WC complex during a circadian cycle of growth in the dark (Brunner and Schafmeier, 2006; Dunlap, 2006). FRQ is required for full *wc-1* photoactivation and also for the translation of the *wc-1* mRNA, resulting in cycles of WC-1 protein in dark-grown mycelia (Cheng et al., 2001b, 2002; Lee et al., 2000; Merrow et al., 2001; Schafmeier et al., 2006). WC-2 is also required for a normal steady-state amount of WC-1. The effect of WC-2 and FRQ on WC-1 is posttranscriptional and requires the formation of a WC complex (Cheng et al., 2002). FRQ activates the expression of *wc-2* (Cheng et al., 2001b, 2002), presumably through inactivation of the WC complex (Neiss et al., 2008), and interacts with the WC complex (Cheng et al., 2001a, 2003b; Denault et al., 2001; Merrow et al., 2001). In addition, the RNA helicase FRH is associated with the WC complex and mediates the interaction between FRQ and the WC complex (Cheng et al., 2005). Interestingly, WC-2 binds rhythmically to the *frq* promoter, in contrast to WC-1, which is constitutively bound, suggesting rhythmic changes in the composition of the WC complex (Belden et al., 2007).

FRQ also plays a relevant role in the regulation of the phosphorylation status of the WC complex and its activity. Lack of FRQ results in hypophosphorylated and transcriptionally active WC complex, while the WC complex is phosphorylated and inactive in the presence of FRQ. The oscillation in the amount of FRQ during a circadian cycle results in changes in the phosphorylation status of the WC complex and its potential activity during the circadian cycle in the dark (Brunner and Schafmeier, 2006). Protein phosphatase 2A interacts with the WC complex in vitro and participates in the dephosphorylation and activation of the WC complex in vivo (Schafmeier et al., 2005). Another phosphatase, PP4, dephosphorylates and activates the WC complex and promotes its nuclear transfer (Cha et al., 2008). On the other hand, the kinases CK-1a and CKII interact with FRQ for the phosphorylation and inactivation of the WC complex (He et al., 2006). The additional observation that CK-1a phosphorylates in vitro WC-2 suggests that this enzyme plays a prominent role in the regulation of the activity of the WC complex (Querfurth et al., 2007). Phosphorylation of the WC complex by casein kinases requires an initial phosphorylation by the cyclic AMP-dependent PKA acting as a priming kinase (Huang et al., 2007). In addition, PKA stabilizes the WC proteins, inhibits WC complex binding to the *frq* promoter in the dark, and inhibits the light function of the WC complex as shown by the enhanced light-dependent accumulation of *al-3* mRNA in a strain lacking PKA (Huang et al., 2007). Phosphorylation of WC proteins by PKA then stabilizes and inactivates the WC complex. The effect of PKA on WC-dependent activation of gene transcription suggests that PKA may have a leading role in the light-dependent phosphorylation of the WC complex that promotes its release from light-regulated promoters. Indeed, protein fractionation experiments have shown that PKA inhibits the nuclear localization of the WC complex (Cha et al., 2008). As mentioned above, PKC negatively regulates WC-1 so that a strain with downregulated PKC has increased levels of WC-1 (Franchi et al., 2005). In contrast, a strain lacking PKA has reduced levels of WC-1 or WC-2 (Huang et al., 2007). Regardless of the effect of these kinases on the amount of WC proteins, reduced activities of either PKA or PKC promote light-dependent gene transcription (Franchi et al., 2005; Huang et al., 2007), suggesting that the role of these two kinases is to inactivate the WC complex.

FRQ thus has a dual regulation on the WC complex during a circadian cycle, inactivating WC activity in the nucleus by regulating its phosphorylation status and supporting at the cytoplasm the nuclear accumulation of the WC complex (Schafmeier et al., 2008). Phosphorylation of FRQ is a relevant step in the transition from a nuclear repressor to a cytoplasmic activator (Cha et al., 2008; He et al., 2006; Schafmeier et al., 2006). The regulation by FRQ of the abundance and activity of the WC complex may explain the changes in the level of photoactivation during the circadian cycle, also known as circadian gating, that have been observed for some *Neurospora* genes (Heintzen et al., 2001; Lauter and Yanofsky, 1993; Merrow et al., 2001).

Another *Neurospora* photoreceptor is VIVID, the product of the *vivid* gene. VIVID is a small protein (186 amino acids) with a single LOV domain that binds the flavin chromophore FAD or FMN and has a prominent role in photoadaptation, the transient activation of gene expression after light exposure (Fig. 1). Strains with mutations in *vivid* exhibit sustained WC-1 light-dependent phosphorylation and a reduction in the amount of WC-1 in the cell, resulting in sustained gene photoactivation and a modified circadian gating response (Heintzen et al., 2001; Schwerdtfeger and Linden, 2001, 2003; Shrode et al., 2001). Unlike WC-1 and WC-2, VIVID is not found in *Neurospora* nuclei and is observed in the cytoplasm only after light induction (Schwerdtfeger and Linden, 2003). Light triggers the formation of a flavin-cysteinyl adduct at the LOV domain with a slow photocycle (about 5 h) (Schwerdtfeger and Linden, 2003). The structure of VIVID has suggested that light triggers a conformational change near the N terminus of the protein after the light-dependent formation of the flavin-cysteinyl adduct (Zoltowski et al., 2007). Detailed studies have shown that VIVID dimerizes within milliseconds of light activation (Lamb et al., 2008; Zoltowski and Crane, 2008). WC-1, WC-2, and VIVID play important roles in the molecular mechanism of the *Neurospora* circadian clock (Brunner and Káldi, 2008; Dunlap, 2006).

The events leading to gene photoactivation can be summarized as follows (Fig. 2). Light reception by the FAD chromophore of WC-1 should trigger the formation of a flavin-cysteinyl adduct, causing a conformational change that leads to WC complex aggregation and promoter binding, chromatin remodeling, and the activation of gene transcription. Gene photoactivation is transient. After further light exposure, WC-1 is phosphorylated, leading to exclusion of the WC complex from the promoter and cessation of transcription. The VIVID protein is required for the transient gene photoactivation, but the mechanism is unknown. The

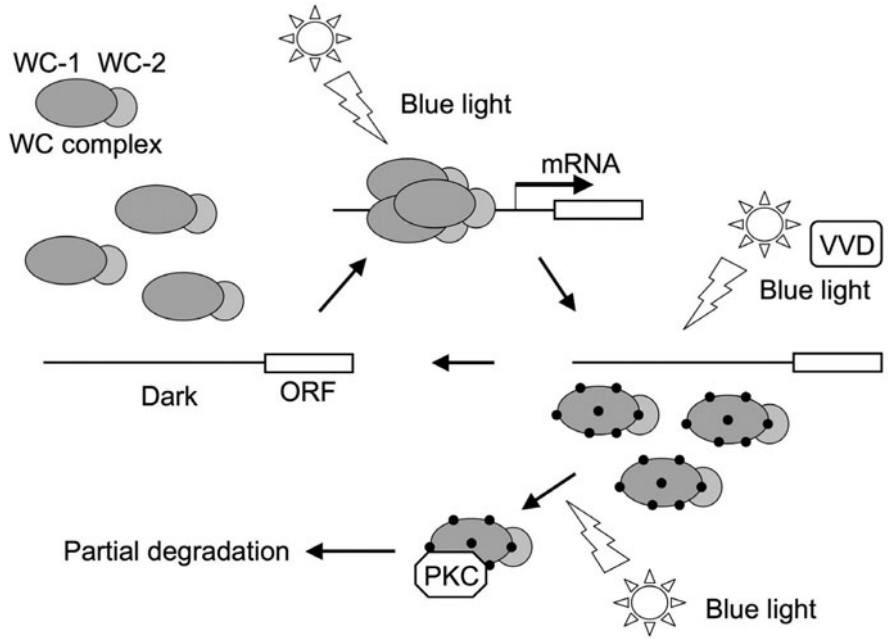

FIGURE 2 A simplified model for the photoactivation of gene expression by the WC complex. The model shows the WC complex with proteins WC-1 and WC-2, and the promoter and ORF of a light-responsive gene. The proteins that interact with the WC complex in the dark (FRQ, RNA helicase FRH, and PKC) are not shown for simplicity. Light reception at the FAD chromophore of WC-1 triggers the formation of a flavin-cysteinyl adduct causing a conformational change that leads to WC complex aggregation and promoter binding, chromatin remodeling (not shown), and the activation of gene transcription. Gene photoactivation is transient. After further light exposure, WC-1 is phosphorylated (black dots) leading to exclusion of the WC complex from the promoter and termination of gene transcription. The protein VIVID (VVD) is required for the transient gene photoactivation, but the mechanism is not known. The excluded WC complex is dephosphorylated and partially degraded, probably through interaction with PKC. The stability, activity, and nuclear localization of the WC complex are controlled by PKA, but the details of this regulation are not known. After a certain period in the dark the WC complex, probably with the addition of newly synthesized WC-1 and WC-2, is ready for gene photoactivation again. Kinases and phosphatases responsible for the light-dependent phosphorylation of WC-1 have not been identified and are not shown.

excluded WC complex is dephosphorylated and partially degraded, probably through an interaction with PKC. The stability, activity, and nuclear localization of the WC complex are controlled by PKA, but the details of this regulation are not known. Since protein phosphatase 2A participates in the dephosphorylation and activation of the WC complex in vivo (Schafmeier et al., 2005), it is possible that this enzyme is also involved in the dephosphorylation of the WC complex after light exposure. After a certain period in the dark, the WC complex, probably with the addition of newly synthesized WC-1 and WC-2, is again ready for gene photoactivation.

WC Proteins in Other Fungi

Genes similar to *Neurospora wc-1* and *wc-2* have been isolated and characterized in ascomycete, basidiomycete, and zygomycete fungi. In many instances, strains with mutations in these genes have reduced photosensitivity, suggesting that the corresponding gene products may play roles similar to those of WC-1 and WC-2 in *Neurospora*. The widespread presence of genes similar to *wc-1* and *wc-2* in fungi suggests that the WC complex and its homologous counterparts represent an ancient photoreceptor system for blue-light sensing in fungi.

Mutations in either *wc* gene of *Aspergillus nidulans*, *lreA* or *lreB*, together with a mutation in the phytochrome gene, prevented full activation of conidiation by light (Purschwitz et al., 2008). The inhibition of sexual structure formation and mycotoxin biosynthesis by light was also lost in strains with combinations of mutations in the *wc* and phytochrome genes, but not in each single mutant, suggesting cooperation of blue- and red-light sensors in *Aspergillus*, which was further confirmed with protein-interaction experiments (Purschwitz et al., 2008).

Two genes with similarity to the *Neurospora wc* genes have been isolated in *Trichoderma atroviride*. The genes, named *blr-1* and *blr-2*, are required for the photoinduction of conidiation and gene expression but not for the light-dependent inhibition of hyphal growth (Casas-Flores et al., 2004; Rosales-Saavedra et al., 2006). In addition, mutants in the *blr* genes are impaired in the induction of conidiation by glucose deprivation (Casas-Flores et al., 2006) and have a high rate of hyphal growth (Casas-Flores et al., 2004), suggesting that the corresponding proteins participate in other cellular pathways that are not regulated by light.

Aerial hyphae development and spore release in the rice blast fungus, *Magnaporthe oryzae*, is regulated by light. Light represses aerial hypha development and spore release, but different wavelengths interact in complex ways to regulate these responses, suggesting the activity of multiple

photoreceptors. Interestingly, a strain with a deletion of the *Magnaporthe wc-1* gene showed aerial hypha development under light exposure and was impaired in spore release, suggesting that light regulation required the *Magnaporthe wc-1* gene product (Lee et al., 2006b). Genes similar to *wc-1* and *wc-2* and *blr-1* and *blr-2* have been isolated and characterized from another plant pathogenic fungus, the ascomycete *Bipolaris oryzae*, which causes brown leaf spot disease in rice. Disruption of these genes revealed that they are essential for conidial development and expression of the photolyase gene after near-UV-light exposure, suggesting that BLR-1 and BLR-2 form a light-sensory system in *B. oryzae* (Kihara et al., 2007; Moriwaki et al., 2008).

A *wc-1* gene has been isolated from two *Fusarium* species, *Fusarium fujikuroi* and *Fusarium oxysporum*. Mutation of the *wc-1* gene in each of these fungi reduced, but did not abolish, light-dependent accumulation of carotenoids, suggesting that the *Fusarium* WC-1 protein is required for full activation of photocarotenogenesis in coordination with other blue-light photoreceptors (Estrada and Avalos, 2008; Ruiz-Roldán et al., 2008). The *F. fujikuroi wc-1* mutant did not show any light-dependent mRNA accumulation for the opsin genes, *car*O and *ops*A, but the mRNA levels increased in the dark, suggesting that WC-1 has a dual role as a repressor and as a light-dependent inducer (Estrada and Avalos, 2009). A reduction in secondary metabolism and conidial production in the *wc-1* mutant of *F. fujikuroi* suggested that this protein plays a major regulatory role in this fungus (Estrada and Avalos, 2008). In addition, the *wc-1* gene from *F. oxysporum* was required for full pathogenicity in mammals (Ruiz-Roldán et al., 2008), a phenotype that had been observed in the *wc* mutants of *Cryptococcus neoformans* (Idnurm and Heitman, 2005a).

The ascomycete *Tuber borchii* is appreciated for the production of truffles, ascocarps developed as symbiotic mycorrhizae with host plants. The *Tuber* homolog of *wc-1* is induced by light, and its protein product may function as a photoreceptor but lacks a polyglutamine activator domain, suggesting that it may not function as a transcriptional regulator (Ambra et al., 2004). In addition, the sequencing of several fungal genomes has allowed the identification of genes similar to *wc-1* and *wc-2* in other ascomycetes (Lombardi and Brody, 2005).

Genes similar to *wc-1* have also been identified in basidiomycete fungi. In *Coprinus cinereus*, a mutant in the *dst1* gene, encoding a protein similar to WC-1, shows a blockage in light-dependent fruiting-body development, suggesting a role for the corresponding protein in light signaling. The mRNA for *dst1* was not observed in vegetative mycelia but was readily detected during the development of *Coprinus* fruiting bodies (Terashima et al., 2005). In another basidiomycete, *Leninula edodes*, the *wc-1* gene was continuously transcribed during fruiting-body development and showed some degree of light regulation (Sano et al., 2007).

Blue light inhibits mating and haploid fruiting in the basidiomycete *C. neoformans*. A strain carrying a mutation in a gene homologous to *wc-1* possesses a blind phenotype, with equal mating in dark and light (Idnurm and Heitman, 2005a; Lu et al., 2005). A gene similar to *Neurospora wc-2* was identified by two independent approaches: sequence homology (Lu et al., 2005) and isolation of an insertional mutant with a blind-mating phenotype (Idnurm and Heitman, 2005a). Overexpression of either *wc* gene resulted in a stronger light-dependent inhibition of mating than that observed for *wc* null mutants (Lu et al., 2005). In contrast to *Neurospora*, the *Cryptococcus* WC-1 protein contains a putative chromophore binding domain but does not contain a Zn finger domain. However, a putative Zn finger is present in the *Cryptococcus* WC-2 protein (Idnurm and Heitman, 2005a; Lu et al., 2005). The *Cryptococcus wc-1* and *wc-2* genes are expressed at very low levels in the dark, and the *wc-2* gene is induced by light (Idnurm and Heitman, 2005a; Lu et al., 2005). In addition, the two WC proteins were shown to physically interact in a yeast two-hybrid assay (Idnurm and Heitman, 2005a). These results suggested that the *Cryptococcus* WC proteins can form a complex that will bind the promoters of light-regulated genes through the WC-2 Zn finger in a mode of action similar to the *Neurospora* WC complex (Idnurm and Heitman, 2005a; Lu et al., 2005).

Perhaps the most surprising observation regarding *wc* genes is the identification of multiple genes with homology to *wc-1* and *wc-2* in the genomes of zygomycete fungi. Three genes similar to *wc-1* (*madA*, *wcoA*, and *wcoB*) and four genes similar to *wc-2* (*madB*, *wctB*, *wctC*, and *wctD*) have been identified and characterized in *P. blakesleeanus* (Idnurm et al., 2006; Sanz et al., 2009). Two of these genes, *madA* and *madB*, are essential for *Phycomyces* photobiology since mutations in *madA* or *madB* reduce the sensitivity to light in *Phycomyces* (Cerdá-Olmedo, 2001). The *mad* mutants were isolated for the defective phototropism of their fruiting bodies (Bergman et al., 1973), and 10 *mad* genes, *madA* through *madJ*, have been characterized (Campuzano et al., 1995; Orejas et al., 1987). The *madA* alleles have mutations in the flavin-binding domain or lack the Zn finger that is presumably involved in DNA binding (Idnurm et al., 2006). The only *madB* allele characterized has a splicing mutation that should produce a truncated protein without the zinc finger (Sanz et al., 2009). The blind phenotype of strains carrying *madA* or *madB* alleles suggests that the flavin-binding domain and Zn finger are both required for *Phycomyces* light responses, including gene photoactivation (Idnurm et al., 2006; Rodríguez-Romero and Corrochano, 2006; Sanz et al., 2009). MADA and MADB interact to form a complex (MAD complex) in yeast two-hybrid assays and after coexpression in *Escherichia coli*, but none of these proteins interact with other *Phycomyces* WC proteins. The absence of any additional interaction between *Phycomyces* WC proteins in yeast two-hybrid assays suggests that the MAD complex must be the main photoreceptor complex in *Phycomyces* (Sanz et al., 2009).

The role of the remaining *wc* genes in *Phycomyces* photobiology is currently unknown (Idnurm et al., 2006; Sanz et al., 2009). However, the functional domains of the WC-1 proteins suggest that they may also serve as blue-light-dependent transcription factors that may be relevant for gene photoactivation. The genes *madA* and *madB* are not induced by light, and the amount of *madA* mRNA is reduced after blue-light exposure. In contrast, *wcoA*, *wcoB*, *wctB*, and *wctD* are induced by light (Idnurm et al., 2006; Rodríguez-Romero and Corrochano, 2006; Sanz et al., 2009). The differential regulation of these genes by light probably reflects different roles for the corresponding proteins in *Phycomyces* photobiology. The *Phycomyces wc-1* genes probably originated after a gene duplication event as shown by the presence of mitogen-activated protein kinase genes upstream of *madA* and *wcoA* and a cyclin gene flanking *wctC* and *wctD* (Idnurm et al., 2006; Sanz et al., 2009).

Three genes with similarities to *wc-1*, *mcwc-1a*, *mcwc-1b*, and *mcwc-1c*, have been identified and characterized in the zygomycete *Mucor circinelloides* (Silva et al., 2006). Like WC-1, the *Mucor* WC proteins have PAS and LOV domains presumably involved in binding a flavin chromophore, but a Zn finger has been predicted only at the carboxyl end of MCWC-1A and MCWC-1C. The fruiting bodies of a strain with a deletion of *mcwc-1a* showed reduced phototropism,

while a strain with a deletion of *mcwc-1c* showed a reduced light-dependent β-carotene biosynthesis, suggesting that MCWC-1A and MCWC-1C may act as photoreceptors for phototropism and photocarotenogenesis, respectively. A strain with a deletion mutation in *mcwc-1b* exhibited normal light responses (Silva et al., 2006), suggesting that the corresponding gene product either plays a secondary role in *Mucor* photobiology or is involved in a light response not yet identified in *Mucor*. However, MCWC-1B is modified by ubiquitylation, presumably to regulate its activity (Silva et al., 2008). The mRNAs for the three *Mucor wc-1* genes are observed in the dark, but only *mcwc-1c* is induced after light exposure. The photoactivation of *mcwc-1c* required an active MCWC-1A, as shown by reduced photoactivation in the *mcwc-1a* mutant (Silva et al., 2006). The genome of the zygomycete *Rhizopus oryzae* contains three *wc-1* genes (Idnurm et al., 2006) and five genes similar to *wc-2* (J. Rodríguez-Romero, personal communication; our unpublished observation). We anticipate that the multiplicity of *wc-1* and *wc-2* genes in zygomycete fungi will lead to specialized photoreceptors for different photoresponses or to operate at different intensity ranges.

Homologs of *wc-1* and *wc-2* have been identified in ascomycete, basidiomycete, and zygomycete fungi. Interestingly, homologs of *vivid* have been described only in ascomycete fungi (Lombardi and Brody, 2005). A gene similar to *vivid*, *envoy*, has been isolated in *Hypocrea jecorina* (*Trichoderma reesei*). The gene is induced by light and by the presence of cellulose, suggesting a connection between light reception and carbon utilization in this fungus (Friedl et al., 2008; Schmoll et al., 2005). Light regulates gene transcription in *Hypocrea* through the activity of the protein ENVOY and the G-alpha subunit GNA3 (Schmoll et al., 2009). However, ENVOY has additional roles in the dark, as shown by the identification of genes regulated by this protein in the absence of light (Schuster et al., 2007). These observations give support to the notion that the *wc-1* and *wc-2* genes and the corresponding WC complex arose early in fungal evolution to serve as a photoreceptor for blue light, while the ancestor of *vivid* arose in the ascomycete lineage, perhaps to serve as a photoreceptor modulating the activity of the WC complex.

Fungal Phytochromes

Phytochromes are photoreceptors that sense red and far-red light through a linear tetrapyrrole chromophore (Rockwell et al., 2006) and are present in plants, fungi, and bacteria (Karniol et al., 2005). First evidence for fungal phytochromes was found in *N. crassa* (Borkovich et al., 2004; Galagan et al., 2003). A phytochrome gene, *fph*A, has been described and characterized in the ascomycete *A. nidulans* (Blumenstein et al., 2005; Brandt et al., 2008). The *Aspergillus* phytochrome has a chromophore binding domain and an output domain composed of a histidine-kinase domain and a response-regulator domain. The FphA phytochrome bound the chromophore biliverdin when it was expressed in *E. coli* and showed the red/far-red light photoreversibility that is typical of phytochromes. In addition, the expressed FphA phytochrome showed a reduced but detectable autophosphorylation. FphA is located in the cytoplasm of germinating spores, and this localization is not changed after red-light exposure. In vivo experiments have showed that FphA molecules interact physically (Blumenstein et al., 2005; Brandt et al., 2008). An *Aspergillus* strain with a deletion of the *fph*A gene showed a reduction in the red-light-dependent inhibition of sexual development and in the red-light-dependent stimulation of asexual development (conidiation). However, lack of red-light sensitivity in the strain without a functional *fph*A gene was not complete, since under red light this strain produced only about 10% of the sexual structures that developed in the dark. This observation suggested that some other red-light-absorbing protein was responsible for most of the red-light repression of sexual development in *Aspergillus* (Blumenstein et al., 2005). The *Aspergillus* phytochrome forms a complex with WC proteins and interacts with the WC-2 homolog LreB in the nucleus, despite the preferred localization of the phytochrome in the cytoplasm (Purschwitz et al., 2008). The interaction between FphA and LreB occurs through the histidine kinase domain and the response regulator domain of FphA (Purschwitz et al., 2009). These results suggest the active transport of the phytochrome to the *Aspergillus* nuclei, perhaps to modulate the transcriptional activity of the WC complex.

Phytochrome genes have been described in other fungi, including the ascomycetes *N. crassa*, *Aspergillus fumigatus*, *Botryotinia fuckeliana*, *Cochliobolus heterostrophus*, *Fusarium graminearum*, and *Fusarium verticillioides* and the basidiomycetes *Ustilago maydis* and *C. neoformans* (Blumenstein et al., 2005; Froehlich et al., 2005; Idnurm and Heitman, 2005a; Karniol et al., 2005). Sequence conservation at the chromophore binding domain and the domain structure of the protein indicate that fungal phytochromes are most similar to bacterial phytochromes (Karniol et al., 2005) and suggest a possible evolutionary origin. However, the role of phytochromes in the photobiology of most fungi is not clear. Deletion of the phytochrome genes in *C. neoformans* (Idnurm and Heitman, 2005a) or *N. crassa* (Froehlich et al., 2005) did not result in a clear phenotype. In addition, a gene for a heme oxygenase, the enzyme required to obtain the phytochrome chromophore from the heme molecule, has not been identified in the genomes of *Neurospora* or *Aspergillus* (Blumenstein et al., 2005; Froehlich et al., 2005), leaving open the question of how these organisms synthesize the chromophores for their phytochrome molecules. The phytochrome genes in *N. crassa* have been investigated in detail. *Neurospora* has two phytochrome genes, *phy-1* and *phy-2*, and the corresponding proteins have the chromophore binding and output domain composed of a histidine-kinase domain and a response regulator domain as described in their *Aspergillus* homolog (Froehlich et al., 2005) (Fig. 1). The amino end of PHY-2 expressed in *E. coli* could bind to the chromophores biliverdin and phycocyanobilin in vitro and showed red/far-red reversibility, suggesting that the *Neurospora* phytochromes should operate as red-light-absorbing photoreceptors. The *phy* genes were not regulated by light, but *phy-1* was regulated by the circadian clock. Phosphorylated and unphosphorylated versions of PHY-1 have been observed in the cytoplasm, suggesting that the mode of action of the *Neurospora* phytochromes should be similar to that of their *Aspergillus* homolog (Froehlich et al., 2005). The observation of two phytochrome genes in *Neurospora* is very striking, since the light responses identified and investigated in *Neurospora* are caused by blue light (Linden et al., 1997a). Red light might be involved in the regulation of the circadian clock. Conidiation in *Neurospora* is governed by a circadian clock that can be entrained by exposure to blue light (Brunner and Schafmeier, 2006; Dunlap and Loros, 2004; Dunlap, 2006; Liu and Bell-Pedersen, 2006). However, the amount of blue and red lights in nature changes throughout the day, with more red light than blue light at dawn and sunset. We can speculate that red-light- and blue-light-absorbing photoreceptors could cooperate to measure the ratio of red light to blue light received by *Neurospora*. Red light is high and blue light is low at dawn, red light decreases throughout the day with a concomitant increase in blue light, and red light increases again as blue light decreases at sunset. It is possible that this photoreceptor cooperation generates two separate inputs into a molecular mechanism that measures more precisely the

time of day for circadian clock regulation. A role for phytochromes as photoreceptors that would allow the detection of red-light variations during the day for circadian regulation has already been suggested (Hellingwerf, 2002). The observation of interactions between red-light- and blue-light-absorbing photoreceptors in *Aspergillus* (Purschwitz et al., 2008) suggests that similar interactions may occur in *Neurospora*. Clearly, the relationship between red light and blue light in circadian clock regulation deserves further investigation.

Red-light effects have been described in some fungi. For example, hyphal growth in *Trichoderma atroviride* is inhibited by light, with both blue and red lights being effective. The red-light inhibition of hyphal growth, which is exacerbated in the *blr* mutants, and the regulation by red light of some blue-light-regulated genes suggested the presence of additional photoreceptors in *Trichoderma* with complex interactions with the BLR photoreceptor (Casas-Flores et al., 2004; Rosales-Saavedra et al., 2006). Red light is also involved in the regulation of spore release in *M. oryzae* (Lee et al., 2006b). It is tempting to speculate that a fungal phytochrome is involved in the regulation of these red-light responses.

Opsins and Cryptochromes

The sequence of several fungal genomes has allowed the identification of new photoreceptor genes, including several genes for fungal rhodopsins (Bieszke et al., 1999a) and cryptochromes (Galagan et al., 2003). Rhodopsins are membrane-embedded seven-transmembrane helix photoreceptors composed of a retinal chromophore bound to an opsin apoprotein. There are two types of rhodopsins, with similar structure but with very little sequence similarity, probably a consequence of their different evolutionary origin. Type II rhodopsins are the photoreceptors for animal vision and bind 11-*cis* retinal as chromophore. Type I rhodopsins bind all-*trans* retinal and serve as ion transporters or sensory receptors in microbes (Sharma et al., 2006; Spudich et al., 2000; Spudich, 2006). Fungal rhodopsins have been described in ascomycete and basidiomycete fungi, and sequence similarities have allowed the distribution of the ascomycete rhodopsins in three groups: a group similar to the *Leptosphaeria maculans* proton-pumping rhodopsin, a group similar to the sensory rhodopsin from *N. crassa*, and a group of opsin-like proteins (Brown, 2004; Brown and Jung, 2006). A lateral gene transfer event from Haloarchaea into the ancestor of ascomycete and basidiomycete fungi has been proposed for the evolutionary origin of fungal rhodopsins (Sharma et al., 2006).

The first fungal rhodopsin gene was identified in *N. crassa*. A *Neurospora* cDNA segment with similarity to archaeal rhodopsin photoreceptors allowed the isolation of the full-length gene *nop-1*. Inactivation of the *nop-1* gene did not result in a clear blind phenotype (Bieszke et al., 1999a) despite the characterization of the NOP-1 protein as having a slow photocycle and long-lived intermediates, consistent with a role as a sensory photoreceptor, not a proton pump (Bergo et al., 2002; Bieszke et al., 1999b; Brown et al., 2001; Furutani et al., 2004) (Fig. 1). The mRNA for the *nop-1* gene accumulates during asexual or sexual development, with a large amount of the mRNA observed late during conidiation, but not during early vegetative growth (Bieszke et al., 1999a, 2007). However, conidiation and sexual development were not altered in the strain with a deletion in the *nop-1* gene (Bieszke et al., 1999a), indicating that the involvement of NOP-1 in *Neurospora* development is minor. A regulatory role for the NOP-1 protein has been suggested based on changes in mRNA accumulation of light and conidiation-regulated genes in mycelia incubated in the dark or exposed to 30 min of white light (Bieszke et al., 2007).

A rhodopsin gene has been isolated from *L. maculans*, the fungus responsible for blackleg disease of *Brassica* species. The *Leptosphaeria ops* gene seems to be constitutively expressed, unlike its *Neurospora* homolog (Idnurm and Howlett, 2001), and is active as a light-driven proton pump when expressed in yeast membranes (Waschuk et al., 2005). The capacity of *Leptosphaeria* rhodopsin to act as a proton pump seems to require specific amino acids and the presence of strongly hydrogen-bonded water molecules (Furutani et al., 2006; Sumii et al., 2005). However, the functional role of a light-dependent proton pump in the biology of *Leptosphaeria maculans* remains to be identified.

Other fungal opsin genes have been inactivated to investigate the functional role of fungal rhodopsins. A gene for an opsin was identified in the genome of the basidiomycete *C. neoformans*, but inactivation did not result in a phenotype (Idnurm and Heitman, 2005a). In the ascomycete *F. fujikuroi*, two opsin genes, *carO* and *opsA*, have been investigated. The gene *carO* was identified in a cluster of genes involved in carotene biosynthesis. The location of *carO* in the *car* gene cluster suggested a common gene regulation. Indeed, the *carO* gene was induced after light exposure and was overexpressed in carotene-overproducing strains, like other neighboring *car* genes (Prado et al., 2004). Since the synthesis of the retinal chromophore requires a regular supply of carotenes, the coregulation of the opsin apoprotein gene with the genes involved in carotenoid biosynthesis may ensure that opsin apoproteins are not wasted in the absence of retinal molecules. The gene *opsA* is not linked to the *car* cluster and is subject to a different regulation: mRNA levels are moderately induced by light, and *opsA* is not overexpressed in carotene-overproducing strains (Estrada and Avalos, 2009). However, the role of the *Fusarium* rhodopsins is not yet known, since gene inactivation did not lead to clear mutant phenotypes (Estrada and Avalos, 2009; Prado et al., 2004).

The absence of obvious phenotypes in fungal strains lacking a functional rhodopsin gene is puzzling. However, the use of action spectroscopy and chromophore replacements with retinal analogues indicated that rhodopsins are the most likely photoreceptors that guide zoospore motility in the chytridomycete fungus *Allomyces reticulatus* (Saranak and Foster, 1997). This suggests a relevant role for this family of photoreceptors in some fungi.

Genes for cryptochromes have also been described in fungal genomes. Cryptochromes are plant blue-light photoreceptors very similar to photolyases, enzymes required for blue-light-dependent DNA repair. However, cryptochromes do not have photolyase activity and bind noncovalently the flavin chromophore FAD and other, probably secondary, chromophores (pterin or deazaflavin). In addition, cryptochromes have a carboxyl end sequence of variable length (Lin and Todo, 2005). There is one cryptochrome gene, *cry*, in the genome of *N. crassa* (Borkovich et al., 2004; Daiyasu et al., 2004), but its function is currently unknown (Fig. 1). The CRY protein is located in the nucleus and the cytoplasm and is regulated by light and the circadian clock. CRY binds the chromophore FAD in vitro, but the deletion of the *cry* gene did not alter photoreactivation repair (Dunlap and Loros, 2005). Sequence similarities suggest that *Neurospora* CRY is a member of the cryptochrome-DASH subfamily (Daiyasu et al., 2004), which has been shown to possess single-stranded DNA-specific photolyase activity (Selby and Sancar, 2006) and act as a transcriptional repressor (Brudler et al., 2003). The single-stranded DNA photolyase activity associated with cryptochrome-DASH proteins suggests that they are probably not involved in photoreception but rather in DNA stability

and repair. However, an additional sensory role for this family of proteins cannot be ruled out.

The genome of *A. nidulans* contains a single photolyase/cryptochrome gene very related to the repair enzymes of the class I CPD photolyase (Bayram et al., 2008a). The *Aspergillus* cryptochrome is located in the nucleus and provides photorepair activity in *E. coli* complementation tests. However, deletion of the cryptochrome gene did not change the sensitivity to UV light in *Aspergillus*, suggesting that this enzyme was not responsible for the major photolyase activity. Instead, the *Aspergillus* cryptochrome mutant exhibited derepressed expression of developmental regulators, promotion of sexual development under inappropriate conditions, and reduced sensitivity to light-dependent inhibition of sexual development, suggesting a sensory role for this protein in the regulation of sexual development by light (Bayram et al., 2008a). In the ascomycete *Sclerotinia sclerotiorum* deletion of the CRY-DASH gene results in minor developmental defects, suggesting that this protein may have a sensory role in this fungus (Veluchamy and Rollins, 2008).

LIGHT TRANSDUCERS: PROTEINS THAT PARTICIPATE IN THE RESPONSES TO LIGHT

Once photons have been received by the photoreceptor, the signal has to be transduced so that the relevant genes and proteins are activated to yield the corresponding photoresponse. Light transduction seems to be reduced to a minimum in *Neurospora* and other fungi using WC-type photoreceptors, since WC operates as a photoreceptor and a transcription factor regulating genes in a light-dependent manner. However, other proteins are thought to participate in the transduction pathway for *Neurospora* photoresponses. Blue light induces the phosphorylation of a 15-kDa protein that was identified as a nucleoside diphosphate kinase encoded by the *ndk-1* gene (Ogura et al., 1999). Mutations in *ndk-1* reduced the NDK-1 activity, prevented the effect of light on the polarity of the perithecial beak (the upward positioning of the end of the sexual structure in this fungus) without affecting perithecial beak phototropism (the orientation of the beak towards light), and showed alterations in other developmental responses and light-dependent carotene biosynthesis. These results suggest that NDK-1 may be an element of the signal transduction pathway for some light responses in *Neurospora* but do not rule out a structural defect in the perithecial beak of *ndk-1* strains (Lee et al., 2006a; Ogura et al., 2001). The observation that NDK-1 is present in the plasma membrane in the dark but relocates to the cytoplasm after light exposure further supports a relevant role for this protein in the light transduction pathway (Yoshida and Hasunuma, 2006). Other signals may also play a role in light transduction. A *sod-1* mutant altered in the enzyme Cu,Zn superoxide dismutase lost the light-induced polarity of perithecia and also showed an enhanced synthesis of carotenes. The results suggested that intracellular reactive oxygen regulated by SOD-1 should have a role in the light transduction pathway (Yoshida and Hasunuma, 2004), a conclusion further supported by the observation of physical interactions between NDK-1 and the catalase CAT-1 in yeast two-hybrid assays (Yoshida et al., 2006).

The *Aspergillus* phytochrome is located in the cytoplasm (Blumenstein et al., 2005), suggesting the need for a transduction pathway to relay the light signal to the nucleus for light-dependent gene regulation. A relevant protein involved in the light transduction pathway in *Aspergillus* is the product of the gene *veA* (Calvo, 2008). A mutation in the *veA* gene allows *Aspergillus* to conidiate in the dark, suggesting a role for the *veA* gene product as a negative regulator of red-light-induced conidiation (Mooney and Yager, 1990). The *veA1* mutant has suffered a nucleotide change in the initiation codon, resulting in a protein 36 amino acids shorter due to the presumed initiation in a secondary methionine codon (Kim et al., 2002). RNA expression and gene overexpression experiments have suggested that the *veA* gene product is also a positive activator of sexual development (Kim et al., 2002). The cellular location of the VeA protein depends on the presence of light. VeA is preferentially located in the nucleus in cells grown in the dark. Light exposure results in a more even distribution of VeA between the nucleus and the cytoplasm, consistent with a role for VeA as a repressor of light-dependent processes. As expected, the mutant VeA1 protein was always found in the cytoplasm regardless of light exposure (Stinnett et al., 2007). Surprisingly, blue but not red light was required to obtain a cytoplasmic localization for VeA (Stinnett et al., 2007), suggesting a possible role for the *Aspergillus* WC complex. Since VeA is involved in red-light-mediated regulation of asexual and sexual development, the blue-light-dependent localization of VeA suggests that red light and blue light interact through the phytochrome and the WC complex to mediate the responses of *Aspergillus* to environmental light. Further results confirmed these proposals. The *Aspergillus* phytochrome, the WC proteins, and VeA were shown to interact in the nucleus, suggesting the formation of a photoreceptor complex that regulates gene transcription after light reception (Purschwitz et al., 2008, 2009). The presence of the phytochrome in the nucleus suggested that this protein was actively transported, perhaps through an interaction with VeA. The VeA protein has been identified as a component of another *Aspergillus* protein complex, the *velvet* complex, with proteins VeA, VelB (a protein similar to VeA), and LaeA (the major regulator of secondary metabolism), which has been proposed to participate in the regulation by light of development and secondary metabolism (Bayram et al., 2008b).

A *Neurospora* strain with a deletion of the *veA* homolog, *ve-1*, produced shortened aerial hyphae and exhibited increased conidiation (Bayram et al., 2008c). Homologs of the *veA* gene have been identified in other ascomycete genomes (Stinnett et al., 2007), suggesting that the corresponding gene product may play similar roles in the light transduction pathways of other fungi. Suppressors of the *veA1* mutations that restored light-dependent conidiation have been isolated (Mooney et al., 1990), and three of them are alleles of the *fluG* gene (Yager et al., 1998), a gene previously identified by mutations resulting in aconidial and fluffy phenotype. Additionally, a mutant strain of *Aspergillus* that conidiates under red or blue light has been used to isolate a mutant that blocked red-light-induced conidiation. Interestingly, the mutant altered in red-light conidiation was yet another allele of the *fluG* gene (Yager et al., 1998). FluG is a protein with limited similarity with prokaryotic glutamine synthetases and is involved in the synthesis of a diffusible, low-molecular-weight factor that controls the initiation of sporulation (Lee and Adams, 1994). The isolation of *fluG* alleles that modified light-dependent conidiation in *Aspergillus* suggested an important role for FluG in the regulation of conidiation by light (Yager et al., 1998). In addition to VeA and FluG, a role for the COP9 signalosome in light regulation of *Aspergillus* development has been suggested (Busch et al., 2003). A *csnD* deletion strain is missing one of the components of COP9 and is blind for light regulation of development, since it produced sexual development in plates grown in light or dark. The results suggested that in *Aspergillus* the COP9 signalosome, which is involved in targeting proteins for degradation, is essential for light-dependent signaling (Busch et al., 2003).

Another protein involved in signal transduction in *Aspergillus* is the protein kinase ImeB, which has been proposed to participate in the regulation of development and secondary metabolism by light (Bayram et al., 2009).

The search for novel mutants in fungal photoresponses may help to identify new proteins involved in signal transduction. In *Cryptococcus* a screening for suppressors of light-dependent inhibition of mating allowed the identification of the *ssn8* gene that is required for transcriptional regulation in yeast (Yeh et al., 2009).

FUNGAL RESPONSES TO LIGHT: PHOTOMORPHOGENESIS

Light regulates fungal development and behavior, presumably for the optimization of spore production and dispersal (Table 1). The main developmental transitions in the life cycle of fungi are spore germination, hyphal growth and branching, and formation of reproductive structures for spore development and dispersal. These developmental transitions are regulated by many environmental factors, including the presence or absence of light (Corrochano and Galland, 2006). The regulation by light of fungal development (photomorphogenesis [Fig. 3]) can be measured precisely, allowing the determination of useful parameters, such as thresholds (Fig. 4).

Effect of Light on Hyphal Growth

Branching in *Neurospora* hyphae is greater in cultures grown in the light than in the dark. As a result, colonies grown in the light are more compact than those grown in the dark (Ambra et al. 2004; Lauter et al. 1998). This effect of light on hyphal growth requires the products of the *wc* genes and has also been observed in the related ascomycete *T. borchii* (Ambra et al., 2004; Lauter et al., 1998). As mentioned above, a similar effect of light has been observed in the ascomycete *T. atroviride*, but contrary to what is observed in *Neurospora*, the light inhibition of hyphal growth did not require the presence of the products of the *Trichoderma wc* genes, and blue light and red light were equally effective (Casas-Flores et al., 2004). Mutations in the *Neurospora* gene *cot-1*, encoding a Ser/Thr protein kinase, result in high branching and colonial growth (Yarden et al., 1992). Two transcription initiation sites in *cot-1* allow the synthesis of two different transcripts. Light stimulates the transcription of the short *cot-1* transcript and represses the synthesis of the *cot-1* long transcript. The ratio of different *cot-1* transcripts is, therefore, regulated by light. The effect of light on *cot-1* transcription depends on the carbon source employed in the culture medium. Sorbose-containing medium stimulates colonial growth and hyphal branching and inhibits the effect of light on *cot-1* transcription (Lauter et al., 1998). However, the amount of COT-1 protein in the cell as measured by specific antibodies is not altered by light (Gorovits et al., 1999), a puzzling observation that suggested that the role of the short *cot-1* transcript and the effect of light on *cot-1* transcription and hyphal branching remain to be elucidated. Another unexplained function for COT-1 has been proposed based on the fact that a *cot-1* mutation suppresses the blind phenotype of *wc-2* mutants. Strains carrying a mutation in the gene *wc-2* and a temperature-sensitive mutation in *cot-1* showed a normal photoactivation of the clock-controlled gene *ccg-1* only when the experiments were carried out at the permissive temperature. This suppression of the *wc-2* phenotype was very specific, since the *wc-2 cot-1* strain did not exhibit photoactivation of *al-3* or *ccg-2* and the *cot-1* mutation did not suppress the blindness of a *wc-1* mutant (Arpaia et al., 1995). The results indicate that a partially active COT-1 kinase might bypass the effect of the *wc-2* mutation in the photoactivation of some *Neurospora* genes (Arpaia et al., 1995). Light also promotes hyphal branching in the plant pathogen *Colletotrichum trifolii* (Chen and Dickman, 2002). The *C. trifolii* gene *tb-3* is homologous to *Neurospora cot-1*.

TABLE 1 Effects of light on fungi

Organism	Phenomenon	Threshold[a]	Reference
P. blakesleeanus	Induction of macrophores	10^{-8} W/m^2	Corrochano and Cerdá-Olmedo, 1990a
	Inhibition of microphores	10^{-4} J/m^2	Corrochano and Cerdá-Olmedo, 1990a
		10^{-10} mol/m^2	Corrochano et al., 1988
	Inhibition of sexual development	3×10^{-2} W/m^2	Yamazaki et al., 1996
	Phototropism	10^{-9} W/m^2	Bergman et al., 1973
		10^{-7} J/m^2	Iino and Schäfer, 1984
	Photocarotenogenesis	10^{-5} J/m^2	Bejarano et al., 1990
		10^{-10} mol/m^2	Bejarano et al., 1990
N. crassa	Induction of protoperithecia	4 J/m^2	Degli-Innocenti et al., 1983
	Circadian clock resetting	10^{-5} mol/m^2	Crosthwaite et al., 1995
	Photocarotenogenesis	30 J/m^2	Schrott, 1980
A. nidulans	Induction of conidiation	135 J/m^2	Mooney and Yager, 1990
		8×10^{-4} mol/m^2	Mooney and Yager, 1990
	Inhibition of sexual development	10^{-4} μmol/m^2 s	Bayram et al., 2008a
T. atroviride	Induction of conidiation	10^{-5} mol/m^2	Horwitz et al., 1990
A. tomato	Inhibition of photoinduced conidiation	10^{-5} mol/m^2	Kumagai, 1989
P. fumosoroseus	Induction of conidiation	1.8×10^{-4} mol/m^2	Sánchez-Murillo et al., 2004
M. oryzae	Suppression of spore release	10^{-5} mol/m^2	Lee et al., 2006b

[a]Light energy is expressed as a fluence rate in watts per square meter for thresholds obtained under continuous illumination or as a fluence in joules per square meter for thresholds obtained after light exposures of a certain duration. The use of monochromatic light allows the measurement of the number of photons that have been applied and the determination of thresholds in moles per square meter for light exposures of a certain duration or in moles per square meter per second for continuous illumination.

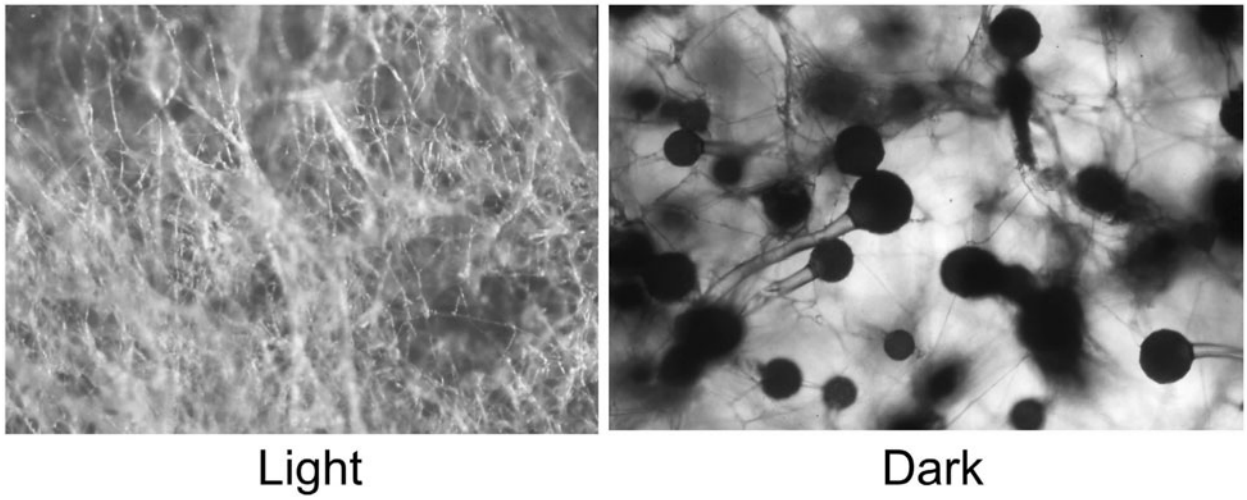

FIGURE 3 Photomorphogenesis in *Phycomyces*: light inhibition of microphore initiation and development in *Phycomyces*. Microphores are short sporangiophores, 1 to 2 mm in length, containing a dark ball on top (sporangia) with matured spores. The fungus was grown under continuous light or in the dark. Microphores appeared only in the mycelial surface of cultures kept in the dark. Photographs by L. M. Corrochano.

Like *cot-1*, *tb-3* is induced by light, but TB3, unlike COT-1, may act as a transcriptional regulator for hyphal branching, as suggested by the nuclear localization of TB3 and the presence of polyglutamine repeats that function as transcriptional activation domains in yeast (Chen and Dickman, 2002). Hyphal growth in the ascomycete *Hypocrea atroviridis* is enhanced by light when the fungus is grown on carbon sources related to cellulose and hemicelluloses. The light effect requires an active blue-light receptor system and additional metabolic signals provided by the growth medium (Friedl et al., 2008).

Regulation of Vegetative and Sexual Reproduction by Light

N. crassa

Conidiation in *Neurospora* is a developmental process that is induced by several environmental cues, such as desiccation, lack of nitrogen or carbon, and the presence of light (Fischer and Kües, 2006; Springer, 1993). Light through the activity of the WC complex is required to obtain the maximum number of conidia, but light results in only a modest fourfold increase in the amount of conidia over that produced by cultures kept in the dark (Lauter et al., 1997). Interestingly, the photoactivation of conidiation is 500-fold greater in a strain with a thermosensitive mutation in the gene *acon-2*. This strain conidiates only at the permissive temperature, 25°C, and the mycelium remains undifferentiated when it is grown at 34°C. At the permissive temperature, the number of conidia developed by the *acon-2* strain is very low, but it reaches the amount produced by the wild type upon exposure to light (Lauter et al., 1997). It seems that a partially functional ACON-2 protein represses conidiation in the dark, allowing light-dependent conidiation to be fully observed.

Other proteins and chemicals may play a role in *Neurospora* photoconidiation. A role for the flavoprotein nitrate reductase in *Neurospora* photoconidiation has been suggested based on the lack of light-induced conidiation in mutants without detectable nitrate reductase activity (Ninnemann, 1991). Additionally, a role for nitric oxide synthase and nitric oxide in *Neurospora* conidiation and its regulation by light has been proposed based on the effect of inhibitors of the enzyme and nitric oxide donors on photoconidiation (Ninnemann and Maier, 1996). The photoinduction of conidiation, like all the *Neurospora* photoresponses, requires the *wc* gene products (Lauter et al., 1997), but an earlier report showed that conidiation of a *wc-1* mutant was promoted by light (Ninnemann, 1991). The different nitrogen sources used in the growth medium may be responsible for the different photoconidation phenotype of the *wc-1* mutant (Lauter et al., 1997). These results suggest a complex interaction of nutritional and environmental factors on the regulation of *Neurospora* conidiation. Conidiation in *Neurospora* is also governed by a circadian clock that can be entrained by light exposure (see chapter 29).

Some genes in *Neurospora* are induced by conidiation and blue light (Carattoli et al., 1995; Lauter and Russo, 1991; Lauter et al., 1992; Lauter and Yanofsky, 1993), and their promoters have an array of developmental, light, and circadian-clock-controlled DNA elements (Bell-Pedersen et al., 1996; Corrochano et al., 1995; Lee and Ebbole, 1998). Mutants altered in the regulation of light-induced genes have been isolated, and some of them have defects in the regulation of conidiation (Carattoli et al., 1995; Linden et al., 1997b; Madi et al., 1994; Navarro-Sampedro et al., 2008). The genes altered in two mutants have been cloned and identified as a general repressor of gene expression (Yamashiro et al., 1996) and a glucose transporter/sensor (Madi et al., 1997), which confirmed the complex relationship between light, nutrient deprivation, and conidiation in *Neurospora*. The regulation of gene expression by light and development are likely to be primary events in the induction of conidiation by light and are a promising avenue of research to unravel the molecular mechanism of *Neurospora* photomorphogenesis.

Sexual development in *Neurospora* is modulated by blue light. The female sexual structures (protoperithecia) of *Neurospora* are induced by blue-light illumination (Degli-Innocenti et al., 1983; Pöggeler et al., 2006). The effect of

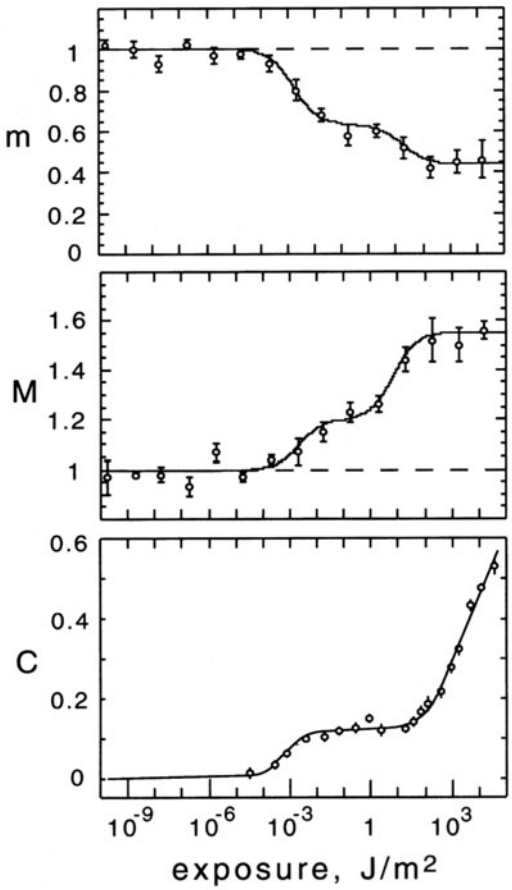

FIGURE 4 Inhibition of microphorogenesis, stimulation of macrophorogenesis, and stimulation of β-carotene accumulation by blue-light pulses. Standard dark cultures, 2 days old, received pulses of blue light of the fluence given in the abscissa. The number of microphores (m) and the dry weight of macrophores (M) were determined 2 days later and given relative to the values found in dark cultures. β-Carotene content was estimated from the absorbance at 452 nm in mycelial samples 12 h after the end of blue-light illumination. The continuous lines represent computer-fitted algebraic expressions (Bejarano et al., 1990; Corrochano and Cerdá-Olmedo, 1990a).

light on protoperithecia formation is enhanced if the culture medium lacks nitrogen. Under these conditions, formation of conidia is strongly reduced. If an inhibitor of DNA methylation (5-azacytidine) is added to the growth medium, conidiation is induced and protoperithecial development is inhibited, suggesting that DNA methylation of regulatory genes impacts the choice of developmental pathways (Kritsky et al., 2002).

A. nidulans

Asexual development in *A. nidulans* is a morphological pathway that culminates with the production of conidia and involves a complex regulatory network of gene regulation and cell differentiation (Adams et al., 1998; Fischer and Kües, 2006). Conidiation in *Aspergillus* is induced by light, but other aspects of *Aspergillus* development are also influenced by light: *Aspergillus* produces hyphae and sexual structures in the dark and mainly conidiophores and conidia in the light. The ratio of sexual to asexual development is changed by light.

The effect of light on conidiation is observed only if light is applied up to 6 h after conidiation has been induced. Surprisingly, only red light is effective, and the stimulatory effect may be reversed if far-red light is applied after a red-light exposure, implicating the action of a phytochrome-like photoreceptor in *Aspergillus* similar to those involved in plant photobiology (Mooney and Yager, 1990). This prediction was later confirmed after the observation of a reduction in the red-light-dependent effect on sexual and asexual development in the phytochrome deletion strain (Blumenstein et al., 2005). However, a detailed spectral characterization of conidiation in *Aspergillus* showed that both blue light and red light were required for full conidial induction, contradicting previous observations that suggested a prominent role for red light only (Purschwitz et al., 2008). Blue light and red light for the activation of conidiation must be sensed by the WC proteins and the phytochrome (in a protein complex described earlier), since only double mutants in the *wc* and phytochrome genes were blind (Purschwitz et al., 2008).

In addition, both blue light and red light inhibit sexual development (Purschwitz et al., 2008), an observation that has been explored fully with detailed stimulus-response experiments with light of different wavelengths (Bayram et al., 2008a). A low-intensity component (10^{-4} to 10^{-2} $\mu mol/m^2 \cdot s$) and a high-intensity component (above 10^{-4} $\mu mol/m^2 \cdot s$) were observed in the inhibition of sexual development after exposure with near-UV, blue, and red lights, suggesting the activity of a complex photosensory system. Deletion of the cryptochrome gene reduced the sensitivity in the near-UV/blue region of the spectrum for the inhibition of sexual development, supporting a role for the *Aspergillus* cryptochrome in the blue-light-dependent inhibition of sexual development (Bayram et al., 2008a). However, the absence of sexual inhibition by light in the phytochrome and *wc* gene double mutants suggested that, in addition to the cryptochrome, the *Aspergillus* WC complex participates in sensing blue light for this developmental response (Purschwitz et al., 2008). No results have been reported about the possible interactions between the cryptochrome and other *Aspergillus* photoreceptors (WC proteins and phytochromes), but the results summarized here and the observation of physical interactions between the phytochrome and the WC proteins (Purschwitz et al., 2008) suggest the possibility of a physical interaction between the cryptochrome and any of the other photoreceptor proteins. The results summarized here suggest that the effect of light on *Aspergillus* development is mediated by the coordinated activity of a cryptochrome (only data for sexual development are available), the WC complex, and a phytochrome, perhaps acting in a coordinated manner in a photoreceptor complex located in the nucleus.

Regulation of Conidiation by Light in Other Ascomycete Fungi

Conidiation in the ascomycete *Trichoderma* is induced by a pulse of blue light (Horwitz et al., 1990) through the action of the two WC proteins BLR-1 and BLR-2 (Casas-Flores et al., 2004). In *T. atroviride*, the gene *phr1* for the DNA repair enzyme photolyase is transiently expressed in mycelia and conidiophores after illumination. The threshold for *phr1* photoactivation is about 10 $\mu mol/m^2$, which is similar to the threshold for photoinduction of conidiation, suggesting that the two photoresponses could use similar photoreceptors (Berrocal-Tito et al., 2000). However, *phr1* photoinduction is not blocked in nonconidiating mutants or in a

photoreception mutant, a result that suggests that gene photoactivation does not require a fully active conidiation pathway and that gene photoactivation and conidial photoinduction may not share all the elements of their respective phototransduction pathways (Berrocal-Tito et al., 1999). In *Trichoderma*, blue light is used as a signal to prevent the harmful effect of UV light by inducing the development of resistant conidia and the expression of the photolyase gene *phr1* (Berrocal-Tito et al., 1999, 2000). A regulatory role for the photolyase has been suggested on the basis of the increased sensitivity of the photoinduction of a reported gene with the *phr1* promoter in the *phr1* deletion strain (Berrocal-Tito et al., 2007).

The ascomycete *Paecilomyces fumosoroseus* is an entomopathogenic fungus that invades insects after conidial contact and germination with the insect cuticle. Blue light has a dual role in conidial production with a stimulatory or inhibitory effect depending on the fluence rate used. When the fungus is grown in the dark, only vegetative hyphae are present. However, 5 min of blue light is enough to induce conidial development (Sánchez-Murillo et al., 2004). A period of competence to light has been observed, since light is effective only when applied between 72 and 96 h of growth. The blue-light threshold is 180 μmol/m^2, and the maximum conidial yield was obtained with 540 μmol/m^2. Higher fluence rates decreased conidiation, suggesting the presence of a complex photosensory system (Sánchez-Murillo et al., 2004).

Regulation of Sporulation and Sexual Development in Zygomycete Fungi: *Phycomyces*

The zygomycete *P. blakesleeanus* (Cerdá-Olmedo and Lipson, 1987; Cerdá-Olmedo, 2001) develops two types of fruiting bodies (sporangiophores) of very different sizes (macrophores and microphores). Blue light stimulates macrophorogenesis, inhibits microphorogenesis (photophorogenesis) (Fig. 3) (Corrochano and Cerdá-Olmedo, 1991, 1992), and has a prominent role in other aspects of *Phycomyces* biology, such as the phototropism of the macrophores and the stimulation of β-carotene biosynthesis in the mycelium (Bejarano et al., 1990; Galland, 1990, 2001).

Sporangiophore development in *Phycomyces* is highly synchronized. Only vegetative mycelium is detected at 48 h, when the mycelium is ready to develop sporangiophores and is sensitive to blue light. Sporangiophores appear soon thereafter and can be easily collected at 72 h. The maximum number of sporangiophores is obtained at 96 h and remains constant for several days. The final numbers of macrophores and microphores in the cultures depend on the blue-light fluence applied at 48 h (Corrochano and Cerdá-Olmedo, 1988, 1990a). The effect of blue light on sporangiophore development follows a two-step stimulus-response curve with thresholds at 10^{-4} and 1 J/m^2, which suggests the presence of different photosystems optimized to operate at different light fluences (Fig. 4) (Corrochano and Cerdá-Olmedo, 1990a).

Since *madA* and *madB* mutants are defective in photophorogenesis (Cerdá-Olmedo, 2001), we can propose that the MAD complex acts as a photoreceptor for the regulation of sporangiophore development. A search for mutants in photomicrophorogenesis, *pim* mutants, identified three mutants with a higher threshold for this photoresponse. The *pim* mutants have normal phototropism and photocarotenogenesis, except for one mutant that showed a higher threshold for photocarotenogenesis (Flores et al., 1998). The *madJ* mutant, blind for phototropism, had a higher threshold for photomicrophorogenesis, while its photomacrophorogenesis and photocarotenogenesis remained normal (Flores et al., 1998). The results suggest the presence of a complex photosensory system with separate transduction pathways for photomicrophorogenesis and photomacrophorogenesis, as already suggested by differences in their action spectra (Corrochano et al., 1988). The presence of multiple *wc-1* genes in the *Phycomyces* genome has given support to the proposal of multiple photoreceptor systems in *Phycomyces* (Idnurm et al., 2006; Sanz et al., 2009).

The molecular basis of photophorogenesis in *Phycomyces* remains largely unknown. Experiments with chemical inhibitors have shown that heterotrimeric G proteins and protein phosphorylation may play a role in the transduction pathway for photophorogenesis (Tsolakis et al., 1999, 2004). Chemical analysis and inhibitor treatments have suggested that pteridines and NO synthase participate in blue-light signaling for photophorogenesis (Maier and Ninnemann, 1995; Maier et al., 2001).

The role of differential gene expression during photophorogenesis in *Phycomyces* was investigated with a method based on PCR with arbitrary primers (Corrochano, 2002). The method allowed the visualization of *Phycomyces* cDNAs differentially expressed during sporangiophore development or after light induction in 48-h mycelia, when the fungus is sensitive to light. A segment of a cDNA from a gene induced by blue light was isolated and identified as a portion of the *hspA* gene encoding the heat shock protein HSP100 (Corrochano, 2002). *hspA* is induced by both light and heat shock, but approximately 10 times more *hspA* mRNA may be observed after a heat shock than after light exposure. This observation suggested that different mechanisms may be involved in the regulation of *hspA* transcription by different environmental cues. In addition, photoactivation of *hspA* is 10^4 times less sensitive than other mycelial light responses, indicating differences in the photoreceptor systems involved. Transcription factors responsible for blue-light-dependent and heat shock-dependent gene transcription may interact in the promoter of *hspA*, as indicated by the identification in close proximity of DNA segments that are present in other heat shock- and light-induced genes (Rodríguez-Romero and Corrochano, 2004). The activation by light of *hspA* gene expression was transient, which suggested the presence of a photoadaptation mechanism similar to that described in *Neurospora* (Rodríguez-Romero and Corrochano, 2006). However, photoadaptation of *hspA* was not prevented by changes in light intensities or dark incubations, unlike photoadaptation in *Neurospora*, suggesting the operation of a different molecular mechanism (Rodríguez-Romero and Corrochano, 2006). Photoadaptation of *hspA* did not prevent further mRNA accumulation after heat shock but correlated with a reduction in MADA photoreceptor mRNA accumulation. The latter result suggests that the presence of less MADA at the *hspA* promoter and the subsequent reduction in mRNA transcription may be responsible for photoadaptation (Rodríguez-Romero and Corrochano, 2006). The activation of *hspA* by light in *Phycomyces* could be required to deal with damaged proteins after exposure to light. HSP100 could also play a role in the phototransduction pathway mediating the disaggregation of regulatory elements. The details of *hspA* photoactivation could give clues to the molecular events involved in sporangiophore development and its regulation by blue light.

Sexual development of *Phycomyces* is inhibited by light. Effective wavelengths were shorter than 490 nm, but the

shape of the action spectra and the most effective wavelength depended on the stage of sexual development. Longer wavelengths were more effective for the inhibition of the final stages of sexual development. In addition, biphasic fluence-response curves were observed under some wavelengths, further confirming the presence of a complex photosensory system for photoinhibition of sexual development in *Phycomyces* (Yamazaki et al., 1996). The threshold for this photoresponse is unusually high for *Phycomyces* (Table 1). The unique shape of the action spectra, with maximum efficiency at 350 to 410 nm, suggests that the photosystems for the inhibition of sexual development may have special features not shared with other photosystems in *Phycomyces*.

Sexual Development in Basidiomycete Fungi

Basidiomycetes are characterized by the complexity of their fruiting bodies, which contain and disperse the spores produced after meiosis. Perhaps as a consequence of this developmental complexity, basidiomycetes show a very complicated pattern of dark and light regulation of several steps of their developmental pathways.

C. *cinereus*

The basidiomycete C. *cinereus* can grow as homokaryon or dikaryon mycelium. The dikaryon is formed after fusion of two homokaryotic mycelia of compatible mating types and develops a fruiting body in which meiosis takes place to produce meiotic basidiospores (Kamada, 2002; Kües, 2000; Wösten and Wessels, 2006). In addition to this sexual cycle, C. *cinereus* develops different types of reproductive and specialized cells: haploid unicellular spores (oidia) develop on oidiophores in the aerial mycelium, and large chlamydospores appear in submerged old mycelium. Hyphal knots are areas of intense hyphal branching that can give rise to globose multicellular bodies (sclerotia) in old cultures or serve as primordia for fruiting bodies. Different environmental conditions determine the developmental pathway of C. *cinereus* with the presence or absence of light serving as the major signal (Fischer and Kües, 2006; Kües, 2000).

The presence of light determines the developmental pathway followed by hyphal knots, since sclerotia are produced in the dark and initiation of fruiting body formation only occurs in the light. Fruiting-body development depends on dark/light cycles, and several steps are influenced by light: hyphal knot formation is inhibited by light; the formation of fruiting body initials, maturation of primordia, and karyogamy are induced by light; and meiosis completion is inhibited by light (Kües, 2000). Mutants altered in different steps of fruiting body development have been isolated, including blind mutants that behave under dark/light cycles as does the wild type grown in the dark. One of the mutant genes has been cloned, and its sequence resembles that of the *Neurospora* gene for the blue-light photoreceptor *wc-1*, suggesting a similar role in C. *cinereus* photomorphogenesis (Terashima et al., 2005).

Of particular interest is the role of light in meiosis, since meiotic progression is controlled by light/dark cycles (Lu, 2000). The timing of karyogamy and meiosis varied in different strains under the same light/dark cycle and depended on the light intensity used. The effect of light was restricted to a period of 16 to 6 h before karyogamy, but a further dark period or low-intensity exposure was required for the completion of meiosis. The suppressing effect of light on the completion of meiosis was not observed in a specific dikaryon due to a mutation in a single gene. The effect of light/dark cycles on meiosis, and possibly on the whole of fruiting-body development, would allow the maturation of fruiting bodies for spore dispersal shortly after daybreak, regardless of night duration (Lu, 2000).

In addition to the role of light on fruiting body development in dikaryons, it is possible to investigate several developmental pathways in C. *cinereus* monokaryons and their regulation by light after the *A* and *B* mating-type pathways have been activated. Monokaryon mycelia develop oidia in high numbers after growth in the dark or in the light. However, a mutant monokaryon with both mating-type pathways activated produced a small number of oidia in the dark and a large amount of oidia when exposed to light (Kertesz-Chaloupkova et al., 1998; Polak et al., 1997). Short illumination times (1 to 2 min) were sufficient to induce oidia above threshold levels, and the effect of light did not spread to nonilluminated mycelia. An exposure of 60 $\mu mol/m^2$ of blue light yielded full oidium induction, with other wavelengths being less effective (Kertesz-Chaloupkova et al., 1998). The role of the *A* activated pathway was further explored after transformation of monokaryons with heterologous and compatible *A*-mating-type genes. The resulting strains showed light induction of oidium production. In addition, light incubation repressed the formation of hyphal knots, sclerotia, and chlamydospores, suggesting a major role for the *A* activated program in development and light regulation (Kües et al., 1998, 2002). This effect was partially modified by an activated *B* pathway (Kües et al., 2002).

REGULATION OF CAROTENE BIOSYNTHESIS BY LIGHT

Accumulation of pigments is a common trait in filamentous fungi. Among them are the carotenoids (Avalos and Cerdá-Olmedo, 2004; Sandmann and Misawa, 2002), terpenoid metabolites that provide the characteristic yellow or orange color of many fungal species. In most cases, carotenoid biosynthesis is induced by light. Well-known examples are the accumulation of the orange neurosporaxanthin by *N. crassa* and *Fusarium* species and the yellow β-carotene by the zygomycetes *P. blakesleeanus* and *M. circinelloides*.

The carotenoid pathways in these fungi coincide in the first steps (Fig. 5), namely, the formation of the colorless phytoene from the condensation of two geranylgeranyl pyrophosphate (GGPP) molecules, a reaction catalyzed by phytoene synthase. Conjugated double bonds are then introduced into this molecule, leading to phytofluene, ζ-carotene, neurosporene, and lycopene in *Phycomyces* and *Mucor* and also to 3,4-didehydro-lycopene in *Neurospora*. Each desaturation shifts the absorption maximum towards longer wavelengths, resulting in the typical yellow to red colors of the desaturation products. The ends of the molecule may be cycled to yield γ-carotene and β-carotene. In *Neurospora* and *Fusarium*, the additional desaturation step, an oxidative cleavage reaction, and a final oxidation lead to neurosporaxanthin formation. The genes for the early steps of the neurosporaxanthin pathway were the first identified for carotenoids in filamentous fungi (Avalos and Cerdá-Olmedo, 2004), thus paving the way for the identification of homologous genes in other fungal species, and facilitating molecular analysis of light effects on their expression.

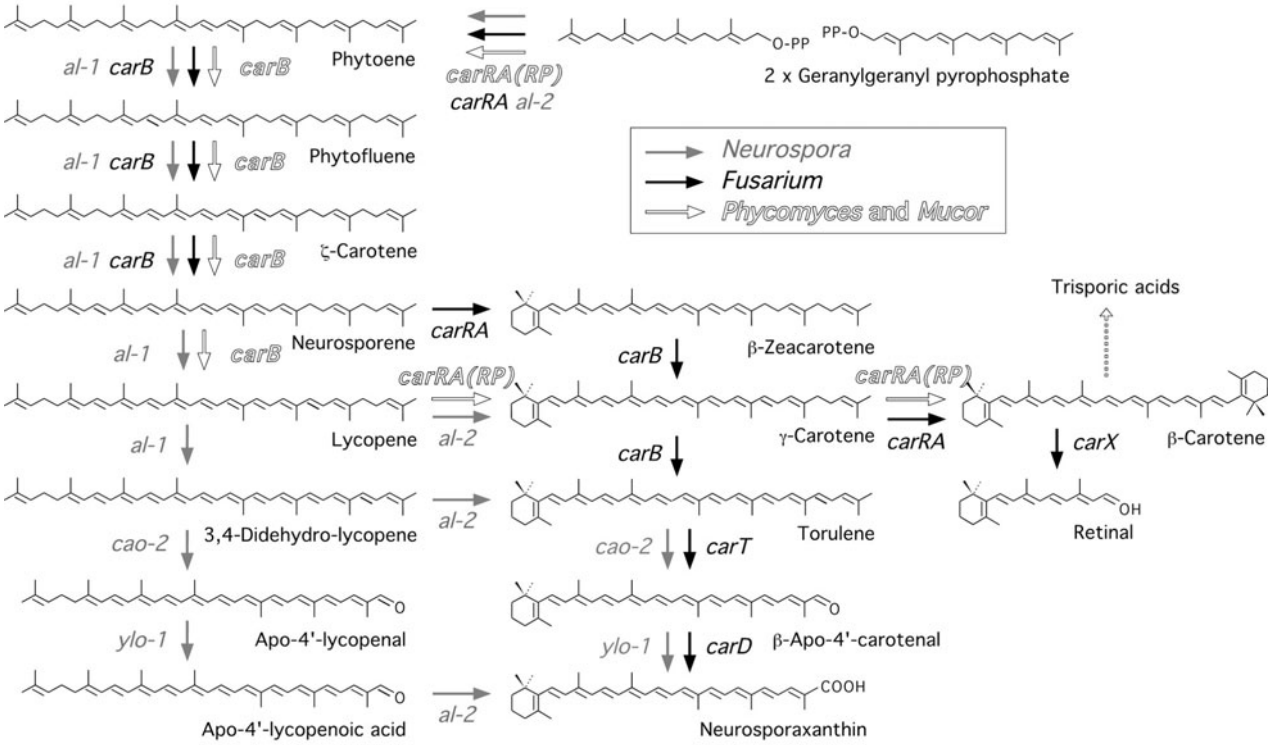

FIGURE 5 Carotenoid biosynthetic pathways in *Neurospora*, *Fusarium*, *Phycomyces*, and *Mucor*. The reactions and genes encoding the responsible enzymes for each species are indicated following the code shown in the box. In contrast to other carotenogenic species, phytoene synthesis from GGPP and cyclization reactions are achieved in fungi by a bifunctional protein, encoded here by the gene *al-2*, *carRA*, or *carRP*. All the genes displayed are regulated by light except *ylo-1*. *carD* has not been investigated. Trisporic acids are apocarotenoid-derived sexual hormones synthesized from β-carotene in zygomycetes.

Ascomycetes: *Neurospora* and *Fusarium*

Light induction of carotenoid biosynthesis was described very early in *Neurospora* research. Exposure of mycelia to light results in the rapid formation of colored carotenoids, which start to accumulate 1 h after light exposure and reach a maximum about 16 h later (Zalokar, 1954). Dark-grown cultures contain significant amounts of the colorless precursor phytoene, which decreases in the first hours of light exposure because of its rapid desaturation to colored products. Very brief illumination times, even less than 1 min, are enough to produce a significant induction of carotenoid biosynthesis (Zalokar, 1955). Similar experiments with *F. fujikuroi* showed a low sensitivity, with a minimal light exposure of 20 min for a significant response (Avalos and Schrott, 1990). The response requires aerobic conditions (Zalokar, 1954), possibly because the photoreceptor needs to be kept in a proper oxidation state (Rau, 1969). Addition of *p*-chloro- or *p*-hydroxymercuribenzoate (Rau et al., 1967) or hydrogen peroxide (Theimer and Rau, 1970) to *Fusarium aquaeductuum* mycelia results in a sustained activation of carotenogenesis in the dark, suggesting that the photooxidation of -SH groups participates in the light induction mechanism in this fungus. Accordingly, the light response was abolished in the presence of strong reducing agents, such as dithionite and hydroxylamine (Theimer and Rau, 1970). However, oxidizing agents had an additive effect with light, suggesting independent mechanisms of action (Theimer and Rau, 1972).

Detailed fluence-response analyses showed a biphasic response: increasing illumination times beyond 16 min resulted in a second rise of carotenoid production (Schrott, 1980). As found with other *Neurospora* photoresponses, mycelia are temporarily insensitive to a second illumination once a first response has been triggered (Schrott, 1981).

Red light does not induce carotenoid biosynthesis in *Neurospora* or *Fusarium* (Schrott et al., 1982), making it unlikely that phytochromes participate in light detection. Interestingly, incubation of *F. aquaeductuum* with the redox dye methylene blue or toluidine blue allowed the accumulation of carotenoids by red light (Lang-Feulner and Rau, 1975), suggesting that these dyes may act as artificial photoreceptors. An initial action spectrum for carotenoid biosynthesis in *Neurospora* showed an efficient carotenoid induction using wavelengths between 400 and 500 nm, with maximal responses between 460 and 480 nm (Zalokar, 1955), which suggested the participation of a flavin as a chromophore in the photoreceptor. This observation was later confirmed by more-accurate action spectra obtained for *Neurospora* (De Fabo et al., 1976) and *Fusarium* (Rau, 1967). For the *Neurospora* experiments, mycelia were incubated in the dark at low temperature after illumination to allow a more efficient photocarotenogenesis (Harding, 1974).

In *Neurospora*, photoinduction of carotenoid biosynthesis is mediated by the WC complex as in other photoresponses (Harding and Turner, 1981) and involves a remarkable rise in the amounts of mRNA for the structural genes *al-1*, *al-2*,

al-3, and *cao-2* (Nelson et al., 1989; Saelices et al., 2007; Schmidhauser et al., 1990, 1994), presumably as a result of their enhanced transcription. An exception is *ylo-1*, responsible for the oxidative reaction of the aldehyde group in the pathway (Fig. 5), whose mRNA levels do not respond to light (Estrada et al., 2008). The induction of the mRNA levels is very fast, reaching a maximum between 15 and 30 min after illumination, and decreasing afterwards to basal levels.

A similar response is exhibited by the structural genes of the carotenoid pathway of *F. fujikuroi*, *carRA*, *carB*, and *carT* (Prado et al., 2004; Prado-Cabrero et al., 2007), in this case extended to a gene for retinal biosynthesis, *carX* (Thewes et al., 2005), and an opsin-like gene (*carO*) (Prado et al., 2004). In agreement with the longer light exposures needed for detectable photocarotenogenesis in this fungus than those needed for *Neurospora*, which have already been mentioned, the maximal mRNA levels are found 1 h after illumination and are also followed by a slow decline. The genes *carB* and *carRA* are clustered with the retinal and opsin genes, *carX* and *carO*. Since retinal is the light-absorbing prosthetic group for opsins, the *car* cluster of *Fusarium* may be contemplated as a set of genes dedicated to the sole purpose of light-controlled production of a functional CarO opsin.

In contrast to the remarkable light regulation of the gene responsible for GGPP synthesis in *Neurospora*, *al-3* (Baima et al., 1991), the corresponding *Fusarium* gene is not photoregulated (Mende et al., 1997). This is not surprising, because this enzymatic step is not exclusive for carotenogenesis and GGPP is used for other purposes, such as protein prenylation (Omer and Gibbs, 1994). Mutations of *al-3* provide an albino phenotype, but only those mutant strains keeping a minimal basal enzymatic activity grow. The mRNA levels for the previous gene in the pathway, the one responsible for the synthesis of the AL-3 substrate farnesyl pyrophosphate, are not controlled by light in either *Fusarium* or *Neurospora* (Homann et al., 1996).

In addition to its regulation by light, carotenoid biosynthesis is coupled to conidiation in *Neurospora* in a light-independent manner (Baima et al., 1992). Although conidium formation is stimulated by light, conidiation is also observed in the dark. As a consequence, cultures of the *wc* mutants have albino mycelia and orange conidiating aerial masses, thus explaining the mutant name.

The effect of light on carotenoid biosynthesis in *Neurospora* is exclusively mediated by the WC proteins. In contrast, targeted mutants of the *wc-1*-like gene found in the *Fusarium* genomes, called *wcoA* in *F. fujikuroi* and *wc1* in *F. oxysporum*, exhibit photoinduction of the carotenoid pathway (Estrada and Avalos, 2008; Ruiz-Roldán et al., 2008). The amount of carotenoids in the light is partially reduced in the *F. oxysporum* mutant, and a qualitative change in the carotenoid mixture was detected in *F. fujikuroi*, explained by a lower transcriptional induction of the gene *carB*, indicating that this WC protein still plays a role in carotenoid photoinduction. However, WC1 and WcoA regulate the light induction of the photolyase gene in *F. oxysporum* and the opsin genes in *F. fujikuroi* (Estrada and Avalos, 2009; Ruiz-Roldán et al., 2008), respectively. Interestingly, *wcoA* and *wc-1* mutants exhibit alterations in light-independent processes, such as secondary metabolism in one case (Estrada and Avalos, 2008) or mice infection in the other (Ruiz-Roldán et al., 2008). These results suggest a recent replacement of WC by another photoreceptor in light regulation of carotenogenesis. The biochemical basis of this photoreceptor is currently under investigation. The occurrence of photoinduction in null *carB* mutants, totally devoid of carotenoids (Fernández-Martín et al., 2000), or in null mutants of opsin genes (Prado et al., 2004; Estrada and Avalos, 2009) argue against the participation of these proteins in photocarotenogenesis. A cryptochrome, for which a single gene is found in the *Fusarium* genomes, could be a plausible candidate. Mutants blocked in carotenoid photoinduction are known in *F. aquaeductuum* (Theimer and Rau, 1969) and *F. fujikuroi* (A. F. Estrada, personal communication), but the mutated genes have not been identified.

Zygomycetes: *Phycomyces* and *Mucor*

Biosynthesis of β-carotene in zygomycetes depends on only two genes, coding for phytoene desaturase and the bifunctional phytoene synthase/carotene cyclase. Both genes are linked and divergently transcribed in *Phycomyces* (Arrach et al., 2001), *Mucor* (Velayos et al., 2000b), and *Blakeslea trispora* (Rodríguez-Saiz et al., 2004), allowing the occurrence of bidirectional regulatory elements in a shared DNA segment for the expression of genes for the complete metabolic pathway.

As in *Neurospora*, fluence-response curves for light-induced carotenogenesis in *Phycomyces* show two components with different thresholds: one at low light intensity and a second one at high light intensity (Fig. 4) (Bejarano et al., 1990). Action spectra for the two components are similar, with maximal efficiencies between 400 and 500 nm (maximum, 450 nm) and a rapid loss of the response beyond 500 nm. The spectra are reminiscent of those observed for *Neurospora* (De Fabo et al., 1976) and *Fusarium* (Rau, 1967) and suggest the participation of a flavin chromophore.

The large collections of *Phycomyces* mutants available include strains with regulatory alterations of carotenoid photoinduction. Among them are the *mad* mutants, already mentioned above. The MADA and MADB proteins are required for normal carotenoid photoinduction (Jayaram et al., 1980), indicating a light-induced activation mechanism mediated by a WC-like complex. In *M. circinelloides* the WC-1 protein MCWC-1C may act as a photoreceptor protein for photocarotenogenesis (Silva et al., 2006). A search of mutants affected specifically in photocarotenogenesis led to the identification of *picA* and *picB* (López-Díaz and Cerdá-Olmedo, 1980), and an unrelated search found defective photocarotenogenesis in a *pim* mutant, affected in photomorphogenesis (Flores et al., 1998). Such strains reveal the occurrence of auxiliary regulatory proteins associated to the major MAD photoreceptor. They could be involved, e.g., in phosphorylation and dephosphorylation steps, as suggested by the effect of inhibitors for protein kinases and phosphatases on carotene production (Tsolakis et al., 1999).

Carotenogenesis in *Phycomyces* is regulated by a feedback mechanism that should be coordinated with photoinduction, but the details remain to be elucidated. According to the current regulation model, β-carotene binds the product of the *carS* gene, CARS, to form a complex that inactivates carotene biosynthesis through an unknown mechanism (Almeida and Cerdá-Olmedo, 2008). Accordingly, the *carS* mutants accumulate large amounts of β-carotene, and the mutants in the structural genes, *carB* or *carR* (R domain of *carRA*), lack β-carotene and consequently accumulate large amounts of phytoene or lycopene, respectively (Fig. 5). Despite their large carotene concentrations, *carB* and *carS* mutants respond to high light intensities with significant increases in their

carotene content (Bejarano et al., 1990). In contrast, *carR* mutants do not respond to light, suggesting the participation of the *carRA* gene in light regulation. Furthermore, mutants of the A domain of *carRA* (known as *carA* mutants, which contain trace amounts of β-carotene) are totally blind. However, these mutants respond to the activation of the pathway by retinol, an apocarotenoid postulated to bind CARS and block its β-carotene binding site. This and other observations led to propose a role for the CARA protein in substrate transfer between the CARB and CARR enzymes that form a multiaggregate complex (Murillo et al., 1981). However, the identification of the A domain of carRA as the one coding for phytoene synthase (Arrach et al., 2001) totally changed this molecular model. Lack of light induction by the *carA* and *carR* mutants suggested a critical role for the CARRA bifunctional polypeptide in light-induced carotenogenesis of *Phycomyces*. As a tentative hypothesis, in addition to the effect at the mRNA level, light could activate the excision of the bifunctional CARRA protein in their active CARR and CARA enzymes.

Transformation experiments of *M. circinelloides* with gene libraries allowed the identification of gene *crgA*, coding for a RING finger protein that acts as a negative regulator of carotene biosynthesis (Navarro et al., 2000). Sequencing and mutation analysis indicated that CRGA acts through a protein-protein interaction mechanism associated to the ubiquitin-proteosome degradation pathway (Lorca-Pascual et al., 2004). The CRGA protein is involved in the modification by ubiquitylation of the photoreceptor MCWC-1B (Silva et al., 2008). Presumably, any of the known mutants giving a similar phenotype in *Phycomyces*, *carS*, *carD*, or *carF* could be affected in a *crgA* orthologue.

Cloning and sequencing of the structural genes in *Phycomyces* and *Mucor* enabled the analysis of the effect of light or other factors on their mRNA levels. The analysis of the first gene available, *carB* from *Phycomyces*, showed a transient photoinduction of its mRNA amounts as fast as the one found in *Neurospora*, but the level of induction was lower (Blasco et al., 2001). Apparent mRNA smears in some samples revealed the great instability of mRNA. Analogous experiments involving *M. circinelloides* showed a similar response but a stronger induction (up to 150 times) (Velayos et al., 2000a), and equivalent results were obtained with the *carRA* orthologous *carRP* (Velayos et al., 2000b). As in *Phycomyces*, the rapid mRNA degradation was visible as a smear for both genes, but in the case of *carB*, the mRNA reduction was observed following a short light pulse (10 s), but not after a long pulse (4 min) (Velayos et al., 2000a).

The complex regulation of carotenogenesis in *Phycomyces* has been recently investigated at the mRNA level for the structural genes of the pathway (Almeida and Cerdá-Olmedo, 2008). The results showed a similar light induction of *carRA* and *carB* mRNAs and no effect of light on the genes required for the early part of the terpenoid pathway, such as *hmgR* and *hmgS*. Light accumulation of *carRA* and *carB* mRNAs was observed to different extents in structural *carA*, *carR*, *carRA*, and *carB* mutants, although a tendency for a lower induction was manifest in the *carR* and *carRA* strains. Similarly, a light induction was clearly observed in the regulatory mutants *carS*, *carF*, and *carD*. However, these mutations did not affect the *carB* and *carRA* mRNA amounts in the dark, suggesting that they have no relation with the light activation mechanism. A manifest induction of *carRA* and *carB* mRNAs was also found upon sexual stimulation, but this induction was additive with the one produced by light (Almeida and Cerdá-Olmedo, 2008).

A close relative of *Phycomyces* and *Mucor*, *B. trispora*, is used for industrial production of β-carotene (Avalos and Cerdá-Olmedo, 2004). Cultures of this fungus under continuous illumination produce fewer carotenes than in the dark (Sutter, 1970). However, later analyses have shown that the genes *carRA* and *carB* respond to light and that a photoinduced carotene accumulation is observed under appropriate light regimes (Quiles-Rosillo et al., 2005).

FUNGAL PHOTOTROPISM: *PHYCOMYCES*

The giant fruiting body (sporangiophore) of *Phycomyces* changes the direction and speed of growth after receiving a number of different stimuli, including light, gravity, wind, touch, and the presence of nearby objects (Cerdá-Olmedo and Lipson, 1987; Cerdá-Olmedo, 2001). The change in the direction of sporangiophore growth after applying unilateral light and the resulting growth towards light is known as phototropism. Phototropism allows sporangiophore growth towards open air for spore dispersal. In addition, an increase or a decrease in ambient light causes changes in the speed of sporangiophore growth (light growth response or photomecism). The responses of the *Phycomyces* sporangiophore to light have been investigated in detail (Galland, 1990, 2001), but modern research in this topic was initiated after Nobel laureate Max Delbrück switched to *Phycomyces* sensory transduction after leaving phage research (Bergman et al., 1969).

The sporangiophore is a single cell, like the rest of the mycelium that is not divided by septae, and responds to the presence of unilateral light by changing the direction of growth. Phototropism, like other responses to light in this fungus, is very sensitive to light, and the sensitivity spans a wide interval of intensities, from 10^{-9} to 10 W/m^{-2} (Bergman et al., 1973), similar to the sensitivity of the human eye (Table 1). This remarkable sensitivity is achieved through the action of two photosystems optimized to operate at different light intensities (Galland and Lipson, 1987). In addition, an adaptation mechanism allows *Phycomyces* to react to changing light intensities for optimal light-directed growth in shading environments (Galland, 1991). Detailed action spectra have suggested that blue light is the most effective and that a flavin chromophore is involved in phototropism, but complexities in the action spectra support the existence of multifaceted sensory systems (Galland, 1983; Galland and Lipson, 1985). Exposure to UV light promotes negative phototropism, a phenomenon initially explained by changes in the focusing effect produced by UV light after interacting with the UV-light-absorbing pigment gallic acid. This theory was discarded after the observation of negative phototropism to UV light in mutants with decreased gallic acid content (Weinkove et al., 1998). The identification of mutants with reduced sensitivity to UV light and the observation that some of the phototropic *mad* mutants are not altered in their response to UV light have given support to the proposal of UV-light photoreceptors in *Phycomyces* (Campuzano et al., 1994, 1996; Martin-Rojas et al., 1995).

The genetic analysis of phototropism was initiated with the isolation of phototropic *mad* mutants (Bergman et al., 1973). To date, only *madA* and *madB* have been identified, and the corresponding protein products form the photoresponsive MAD complex described earlier (Idnurm et al.,

2006; Sanz et al., 2009). None of the remaining *mad* mutants have mutations in any other *wc* gene, suggesting that the corresponding proteins may act in the signal transduction pathway (Sanz et al., 2009). Other *mad* mutants (*madD* and *madE*) are altered in sporangiophore responses to light and other stimuli, which suggested that the corresponding genes were required for the regulation of sporangiophore growth (Bergman et al., 1973). However, detailed phenotypic characterization has suggested an interaction between light and other environmental signals in the regulation of sporangiophore growth (Campuzano et al., 1996).

Mutations in *madB* and *madC* change the sensitivity to light and produce major changes in the action spectrum for phototropism, unlike mutations in *madA* that only reduce the sensitivity to light (Galland, 1983; Galland and Lipson, 1985). Mutations in *madC* are very specific, since they modify only the responses of the sporangiophore to light without altering mycelial photoresponses (Bergman et al., 1973; Corrochano and Cerdá-Olmedo, 1990b), and the corresponding gene product may represent a novel photoreceptor specific for the sporangiophore. It is possible that some of the additional WC-1 proteins participate in phototropism as secondary photoreceptors to provide the wide sensitivity that is characteristic of this fungus.

It is interesting that the mechanism of action of the MAD complex in a short-term phototropic photoresponse is not very clear. The *Phycomyces* fruiting body is a cylinder composed of a single cell several centimeters in length, and phototropic bending is observed 5 min after applying unilateral light (Galland, 1990), a short time for a light-dependent transcription factor to operate efficiently through gene transcription and protein translation. Yet mutant versions of MADA lacking the Zn finger show reduced photosensitivity (Idnurm et al., 2006), suggesting that MADA should operate in the *Phycomyces* fruiting body through asymmetric light-dependent gene transcription and translation. In addition, measurements of light profiles through the sporangiophore and theoretical calculations have suggested that phototropism is controlled by dichroic photoreceptors located at the vicinity of the plasma membrane (Fukshansky, 1993). Nuclei are positioned near the plasma membrane in this multinucleated syncytium, which may account for the biophysical localization of the presumptive photoreceptor. The prominent role of MADA in *Phycomyces* phototropism suggests that these proteins may operate through a novel mechanism not yet described for WC-1 and WC-2 in *Neurospora*.

Phototropism has been described and investigated in other fungi. The perithecial beaks of *Neurospora* exhibit positive phototropism when illuminated with blue light, but perithecia from cultures kept in the dark form randomly oriented beaks (Harding and Melles, 1983). The photoreceptor for this response is the WC complex, since mutations in *wc-1* and *wc-2* prevent perithecial beak phototropism (Harding and Melles, 1983). The small fruiting bodies of *Mucor* grow towards blue and green light, and the blue-light photoreceptor MCWC-1A is required for this response (Silva et al., 2006). Phototropism of the fruiting bodies (sporangiophores) of several species of the zygomycete *Pilobolus* has been investigated in some detail. Not only do sporangiophores grow towards blue light, but also spore-containing sporangia are then shot towards the light source in order to promote better spore dispersal (Page, 1962). It is very likely that a photoreceptor similar to WC-1 is involved in *Pilobolus* phototropism.

SUMMARY

Fungi use blue light as the primary signal for photoreception and the WC complex as the primary photoreceptor system for blue light. There are exceptions to this paradigm, such as the phytochrome-mediated red-light reception in *A. nidulans*. However, the ubiquity of blue-light responses in fungi and the relevant role in blue-light reception of proteins similar to *Neurospora* WC-1 and WC-2 in ascomycete, basidiomycete, and zygomycete fungi strongly argue in favor of the WC complex as an ancient and widespread blue-light receptor in fungi. Since LOV, the WC-1 flavin-binding domain, is the flavin-binding domain of photoreceptors in plants (Christie, 2007) and bacteria (Losi, 2004), we can speculate that LOV represents an ancient domain for flavin binding that has been shared by different organisms for sensing blue light.

The initial effect of light is to activate gene transcription, presumably after the activation of the WC complex. Other active photoreceptors are located in the nucleus, and the identification of physical interactions with the WC complex suggests that the signals from the photons received by these photoreceptors are transduced to the WC complex to regulate gene activation. After induction of gene transcription, the increase in the corresponding proteins will activate the cellular pathway that is regulated by light. These may be developmental or metabolic pathways, as described above for the biosynthesis of carotenoids. It is possible that a more sophisticated signal transduction pathway operates in phototropism due to the fast response time and the spatial properties of the asymmetrical response.

Fungal photobiology provides a unique opportunity to investigate the effect of light on a wide group of microbial eukaryotes without the complexities related to photosynthesis and other energy-oriented light perception mechanisms. Uncovering the mechanisms of action of fungal photoreceptors will help us to understand how fungal cells receive and transduce the energy contained in photons to obtain information about the surrounding environment and modulate their development and pattern of growth.

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Circadian Rhythms

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INTRODUCTION

The 24-h rotation of the Earth about its axis imparts a predictable daily cycle in environmental factors, such as the light/dark cycle, which organisms living on or near the surface must cope with and/or utilize. To exploit the opportunities and meet the challenges that arise from rhythmic changes in the environment, most organisms have evolved an internal timing mechanism that enables them to anticipate and prepare for these predictable cycles in their surroundings. This timing mechanism was termed "circadian" in 1959 by Franz Halberg, derived from the Latin *circa*, about; and *dies*, a day. Circadian clocks are biological systems that measure the passage of time and confer daily rhythms in endogenous processes that impact an organism's internal biology, physiology, and behavior. Although circadian clocks are not essential for an organism's survival, there is strong evidence that clocks provide a survival advantage to organisms (DeCoursey, 1961; Ouyang et al., 1998; Woelfle et al., 2004).

Circadian clocks exist at all levels of life and are found in organisms as diverse as unicellular prokaryotic cyanobacteria, plants, fungi, insects, and humans. Within these organisms, circadian systems regulate rhythms at many different levels, including gene expression, hormone production, and even the sleep/wake cycles of an entire organism. Because the impact of the clock in humans is far-reaching, it is not surprising that abnormalities in circadian timing have significant effects on human health. Defective clocks are associated with sleep disorders, epilepsy, cerebrovascular disease, multiple sclerosis, headaches, cardiovascular malfunction, and cancer (Oklejewicz et al., 2008; Otsuka et al., 2001; Pace-Schottand Hobson, 2002; Turek et al., 2001). A correlation has been established between sleep disorders and alterations in genes involved in circadian clock function (Xu et al., 2005; Toh et al., 2001). A role of the circadian clock in cell division and cancer has also been described (Pregueiro et al., 2006; Fu and Lee, 2003; Matsuo et al., 2003), and there is a known relationship between the times of day that chemotherapeutic agents are administered and the efficiency and/or toxicity of these drugs (Wood and Hrushesky, 1996; Mormont and Levi, 2003). These examples emphasize the importance of studying the molecular organization of the circadian system, as a thorough understanding of the clock at the molecular/biochemical level will provide new opportunities for the development of therapies for diseases and conditions related to circadian pathologies.

Significant strides have been made in unraveling the mechanism of the clock in several model organisms, including *Neurospora*. In mammals, the circadian system is the product of cross talk between several different intercellular and intracellular oscillators (Aton and Herzog, 2005). However, because the core mechanism of the clock in organisms lies within the cell, microbial organisms such as *Neurospora* provide important models for the dissection of an inherently complex network to address questions about the mechanism of the clock and the role of the environment and metabolic state of the cell on oscillatory behavior.

A Historical Perspective of Chronobiology

A French astronomer by the name of Jean Jacques d'Ortuos de Mairan is credited with performing the first experiment in chronobiology (i.e., the field of biological rhythm studies). In 1729, de Mairan noticed that the leaves of a *Mimosa* plant continued to remain open during the daylight hours and closed during the night even when the plant was isolated from the daily light/dark (LD) cycle thought to drive such rhythms (de Mairan, 1729). At that time, and for many years after, most scholars believed that the continuing rhythm of leaf movement and other rhythms described in biological systems were a result of some environmental

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cue (zeitgeber [German for time-giver]) that was either uncontrolled (such as gravity) or unidentified (Somers, 1999; Moore-Ede et al., 1982).

One hundred years after de Mairan's discovery, another Frenchman, Augustin de Candolle, was among the first to demonstrate the truly endogenous nature of the plant circadian rhythm. Candolle reported that the period (defined as the duration of one complete activity cycle) of the rhythm of leaf movement in the *Mimosa* plant was, in fact, not exactly 24 h, but rather 22 to 23 h in constant darkness (DD) (Somers, 1999; Moore-Ede et al., 1982). This observation demonstrated that the leaf movement rhythm was not simply driven by the 24-h environmental cycle. The importance of this result would go unrecognized for another 100 years.

Throughout the 1950s Bünning, Aschoff, and Pittendrigh laid the foundation for the modern field of chronobiology. No longer were these rhythms just curiosities that were observed in some species of plants; they became a bona fide biological phenomenon worthy of serious study. Circadian rhythms were soon discovered and described in many organisms, including *Gonyaulax*, *Neurospora*, *Drosophila*, and mice (Pittendrigh, 1993). In 1960, the first biological clocks conference was held at Cold Spring Harbor, and a second Cold Spring Harbor conference was held recently, in 2007 (Biological Laboratory, 1960; Stillman et al., 2007).

What Defines a Circadian Rhythm?

There are three cardinal properties that define circadian rhythms (Fig. 1 through 3). First, the period length of circadian rhythms is, by definition, close to 24 h. Period generation is endogenous to the organism and self-sustaining, such that it occurs in the absence of environmental cues and is not simply driven by external stimuli. The period exhibited under constant conditions is termed the free-running period (FRP), and its value can vary between species and between individuals within a species.

The second characteristic of circadian rhythms is that the rhythms are entrained to environmental signals such as light and temperature cycles (Fig. 2). This is an important feature, because as the FRP is close to 24 h, the clock must be reset each day in order to avoid falling out of alignment with the external 24-h environmental cycle. Thus, circadian systems must sense and respond to external stimuli to adjust their cycle length to that of the environmental cycle. This coupling process is facilitated by the sensitivity of the circadian clock to environmental cues, such as light and temperature. Crucially, circadian clocks show differential sensitivity to zeitgebers during the course of a day. For example, in many diurnal organisms that use light as an entraining signal, light exposure in the early subjective night produces a delay in the phase of the clock, whereas light in the late subjective night causes a phase advance. Light treatment during the subjective day causes little or no change in the phase of the clock. In other words, the responses to zeitgebers, such as light, can be controlled by an organism's sense of time. The magnitude and direction of the response to a zeitgeber can be plotted with respect to an organism's own circadian phase at the time that the environmental cue was given. Such a plot is called a phase-response curve (Fig. 3) (Johnson et al., 2003). Studies of the molecular response of the clock to light in *Neurospora* was critical to our understanding of how the same light pulse given at different times of day can elicit different responses in the organism (see below).

Thirdly, circadian rhythms are temperature compensated such that the period length of the rhythm is relatively constant at different physiologically relevant temperatures. This property is in contrast to most biochemical reactions, which double in rate with a 10°C temperature increase. Temperature compensation gives the clock reliability in an organism's natural environment, despite the changes that occur in ambient temperature. Although the mechanisms of temperature compensation are not well understood, it is expected that while individual reactions within the clock are affected by changes in temperature, the system as a whole is somehow buffered against changes in FRP. Temperature compensation of circadian clocks does not imply that circadian clocks are temperature insensitive. As mentioned earlier, temperature can be as strong a zeitgeber as light in entraining biological clocks (Liu et al., 1998).

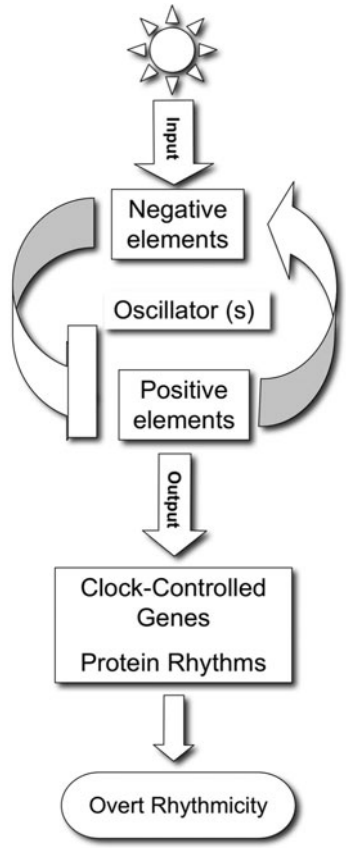

FIGURE 1 Circadian clock properties. Flow of information in the circadian system. See the text for details.

To measure endogenously generated circadian activities, as opposed to rhythms that may be driven by environmental signals, the rhythms are measured under constant environmental conditions, such as in constant light (LL) or DD at constant temperature. Under these conditions, time is measured as endogenous, or circadian time (CT). The CT "hour" is calculated by dividing the FRP by 24 h. Under constant conditions, the subjective day begins at CT0 (subjective dawn), whereas CT12 represents subjective dusk.

The Circadian Clock Is a System That Links Environmental Cycles to Rhythmic Behavior

The ubiquity of circadian systems has allowed for their characterization in a broad range of model organisms.

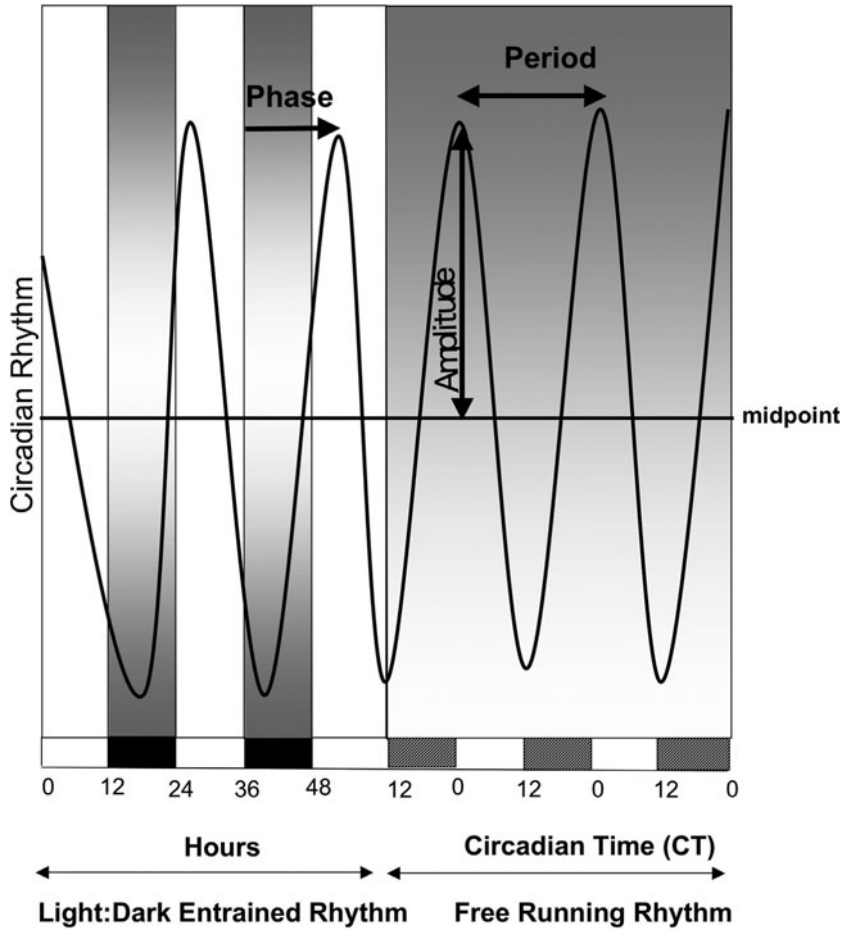

FIGURE 2 Properties of a circadian rhythm. From left to right, the rhythm is entrained to a 12-h LD cycle and then released into DD. In the entrained cycle, the dark boxes represent night, and the light boxes represent day. In the free-running rhythm, the hatched boxes represent subjective night. Period, phase, and amplitude are indicated and are described in the text.

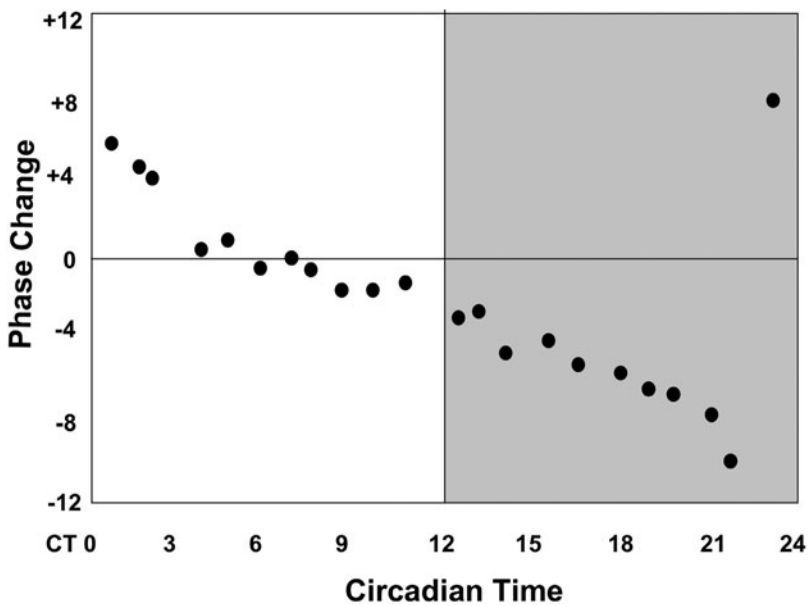

FIGURE 3 Plot of a representative phase-response curve in *Neurospora* to a light 30-min light pulse given at the indicated circadian time (CT) (*x* axis) (Dharmananda, 1980). The change in phase compared to cultures that were not given a light pulse is plotted. Positive numbers represent a phase advance, and negative numbers represent a phase delay in the developmental rhythm.

While individual clock genes and proteins can vary among phylogenetically diverse organisms, the overall organization and underlying mechanisms of each clock system appear to be highly conserved (Bell-Pedersen et al., 2005). At the core of every biological clock system is an autonomous, entrainable oscillator (Fig. 1). An oscillator is defined as a system of components whose interaction produces a rhythm with a definable period length. A circadian oscillator is a set of components that act together to produce a circadian rhythm. Circadian oscillators in eukaryotes are comprised of both positive and negative elements with opposing functions: positive effector proteins stimulate expression of the negative effector(s), which acts to inhibit the activity of the activating positive elements. The net effect is an autoregulatory, negative-feedback loop in which the negative element ultimately inhibits its own expression. The interplay of these elements results in an oscillation with a period near 24 h. A circadian oscillator is a "pacemaker" if it is able to sustain its own oscillations and is able to entrain rhythmic outputs and other oscillators that might be present in an organism. Input pathways feed time-of-day information from the environment into the oscillator or pacemaker so that the circadian clock is synchronized to local time. Output pathways relay the time-of-day information from an oscillator to downstream effectors, thus ensuring that the proper processes occur at the correct time of day.

CIRCADIAN RHYTHMS IN *NEUROSPORA CRASSA*

In 1953, a pattern in the growth of *Neurospora* during LD cycles or in DD was reported (Brandt, 1953); cultures grown in either of these conditions alternated between vegetative (mycelial) growth and differentiated aerial hyphae, which form the asexual conidiospores. In 1959, Pittendrigh and colleagues published the first paper demonstrating that the developmental rhythm is controlled by an endogenous, temperature-compensated circadian clock (Pittendrigh et al., 1959). While the observed rhythms continued for some time in DD, they had a tendency to dampen (decline in amplitude) over the course of an experiment due to a buildup in CO_2 levels in the growth tube (called race tubes; see Fig. 4 for a description of the assay). Passing sterile air through the tubes alleviated this problem, but this was cumbersome. The subsequent isolation of the *band* (*bd*) mutation streamlined the process of circadian rhythm analysis in *Neurospora* as the mutation confers resistance to the elevated CO_2 levels without affecting the underlying circadian rhythm (Sargent and Kaltenborn, 1972). The FRP of the conidiation rhythm in *bd* mutants was found to be about 22.5 h in DD at 25°C, and this mutant is still used as the laboratory "wild-type" strain for circadian rhythm research. Only recently was the *bd* gene cloned and shown to be *ras-1* (Belden et al., 2007). The *bd* mutation itself is a dominant allele of *ras-1* and is thought to influence conidiation by influencing ROS (reactive oxygen species) levels within *Neurospora*. Indeed, the addition of ROS-generating prooxidants to the growth medium phenocopies the effects of the *bd* mutation on circadian banding (Belden et al., 2007).

One of the scientists who worked with Colin Pittendrigh to demonstrate the circadian nature of the *Neurospora* conidiation rhythm was Victor Bruce. The year after the *bd* strain was isolated, the first mutations affecting period length in *Neurospora* were obtained by one of Bruce's former students, Jerry Feldman (Feldman and Hoyle, 1971). The mutations he obtained, which he dubbed *frequency* (*frq*), had FRPs ranging from 16.5 to 29 h (Gardner and Feldman, 1980; Feldman and Hoyle, 1973) (recall that the wild-type FRP in *Neurospora* is 22.5 h). Some of these mutations (i.e., the 29-h long period frq^7 allele) were also found to have an effect on temperature compensation of the clock (Gardner and Feldman, 1981). It was later determined that the *frq* locus encodes a key component of the core *Neurospora* circadian oscillator (Aronson et al., 1994b).

Characterization of the *Neurospora* clock and its overt rhythms proceeded more slowly during the 1980s. During this time, much effort was focused on describing the physiological basis of the clock, and the effects of various drugs and chemicals were examined (Nakashima, 1986; Nakashima et al., 1981; Schulz et al., 1985). Cultures of *Neurospora* were even taken into outer space to definitively show that the developmental rhythm is not a result of some exogenous cue resulting from the Earth's rotation (Ferraro et al., 1989; Sulzman et al., 1984; Sulzman, 1984), but little progress was made in determining the true nature of the molecular oscillator.

In the mid-1980s a new generation of *Neurospora* chronobiologists emerged on the scene equipped with many of the molecular biology techniques used today. One key event that led the way to determining the basic molecular workings of the circadian oscillator in *Neurospora* was the cloning of *frq* by the Dunlap lab (McClung et al., 1989).

In 1989, the first two clock-controlled genes (ccgs) (genes that are rhythmic but lie downstream of the oscillator mechanism) were identified through a subtractive hybridization assay (Loros et al., 1989). By 1991, it was demonstrated that the clock regulates these genes at the transcriptional level (Loros and Dunlap, 1991), which provided the first insight into a mechanism for clock-controlled gene expression. Until 1996, however, it was thought that the clock in *Neurospora* served only to regulate development; the most robust rhythm observed was that seen in conidiation, and both ccgs isolated up to that point were also induced in response to light and development (Bell-Pedersen et al., 1992; Arpaia et al., 1995). A different screen isolated six more ccgs; among them was the previously cloned copper metallothionein gene (*cmt*) involved in copper transport and detoxification (Bell-Pedersen et al., 1996a). In addition to *cmt*, two of the other newly found ccgs were not transcriptionally induced during conidiation. This discovery provided evidence to support the idea that the *Neurospora* clock system regulates processes other than development.

A complete picture of the central oscillator had emerged by 1997. Studies thus far had implicated a regulatory feedback loop in the generation of circadian oscillations (Dunlap, 1996). Inactivation of *frq* in *Neurospora* by point mutation resulted in high-level expression of the corresponding gene, and forced overexpression of *frq* resulted in reduced expression of the endogenous gene. Together these data revealed that the FRQ protein functions as a "negative element" in the circadian oscillator (Aronson et al., 1994a). It had been known for some time that the conidiation rhythm was responsive to light (Sargent and Briggs, 1967) and that the *wc-1* and *wc-2* genes and protein products were required for this, as well as all other known light responses in *Neurospora* (Russo, 1988). Cultures of *wc*-null mutant strains are arrhythmic. This was thought to be due to the inability of *wc* mutant cells to perceive light and thus undergo synchronization of the cellular oscillators following an LL-to-DD transfer. The turning point came when it was demonstrated that the products of both *wc-1* and *wc-2* are necessary for robust expression of *frq* transcripts, even in

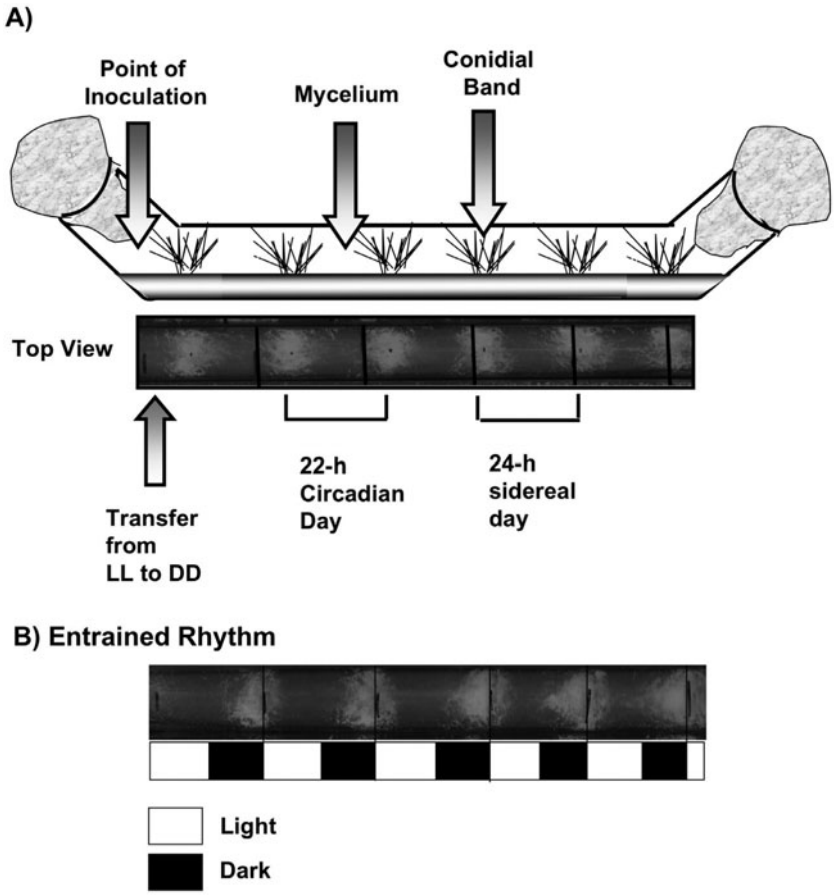

FIGURE 4 Assays used to examine rhythmicity in *Neurospora*. (A) The race tube assay. In *Neurospora*, the developmental rhythm is typically assayed in hollow glass tubes that are partially filled with a solid medium, although petri dishes or capped, horizontal 150-mm test tubes can also be used (Lakin-Thomas et al., 1990). Cultures of mycelia or conidia are inoculated at one end of the tube and incubated in LL for about a day. The growth front is marked, and the culture is transferred to DD to monitor the free-running rhythm (A) or can be maintained in an environmental cycle, such as an LD cycle (B) to monitor an entrained rhythm. In constant conditions, the growth front is marked every 24 h, and in the 12-h LD cycle, the race tube is marked at lights on. Under constant conditions, the developmental switch for the production of conidiophores is activated in the late subjective night of the circadian cycle, resulting in the production of conidial spores (conidial band) for a defined part of the day. Once the asexual pathway is initiated, this developmental process proceeds at a rate dependent on factors that are independent of the clock, including culture and strain type. The signal for development is switched off some time later in the middle of the subjective circadian day, and undifferentiated filamentous mycelia again predominate. Note that in DD, the period of the rhythm is shorter than 24 h, but in the 12-h LD cycle, the period is exactly 24 h.

dark-grown cultures (Crosthwaite et al., 1997). This finding marked the identification of the first "positive elements" in any circadian oscillator, and they were PER-ARNT-SIM (PAS)-PAS heterodimers, as have been all of the positive elements identified subsequently in animal clocks. Indeed, extended sequence similarity among these positive elements in eukaryotes suggests a common ancestral gene, with WC-1 forming the root of the evolutionary tree (Lee et al., 2000; Tauber et al., 2004).

THE *NEUROSPORA* FRQ/WCC OSCILLATOR

The *frq*, *wc-1*, and *wc-2* genes and the interactions of their protein products, as well as other accessory proteins that function to modify the activity or stability of the key components, constitute the circadian FRQ/WCC oscillator in *Neurospora* (Table 1). Deletion of *frq*, *wc-1*, or *wc-2* results in a loss of circadian regulation of conidiation under standard growth conditions in DD (Aronson et al., 1994b; Collett et al., 2002; He et al., 2002; Lee et al., 2003; Crosthwaite et al., 1997). Point mutations in *frq* or the *wc* genes result in a variety of phenotypes including altered period length (from 16 to 35 h) and arrhythmia and/or affect temperature compensation of the circadian rhythm (Yang et al., 2002; Liu et al., 2000; He et al., 2005b; Collett et al., 2001; Aronson et al., 1994a). Constitutive expression of *frq* mRNA, which normally accumulates with a period equal to that of the conidiation rhythm, also results in arrhythmia (Aronson

TABLE 1 Known components of the *Neurospora* FRQ/WCC oscillator[a]

Protein component	Clock-associated function
FRQ	An essential clock component that binds to itself and to FRH to form a complex that functions as the negative element via repression of WCC activity.
WC-1	An essential clock component and a blue-light photoreceptor that binds to WC-2 to form the WCC; functions as the positive element in the molecular feedback loop by binding to the *frq* promoter and activating *frq* transcription; and controls some aspects of rhythmic output. The WCC complex is also required for light resetting of the clock.
WC-2	An essential clock component that binds to WC-1 to form the WCC; functions as the positive element in the molecular feedback loop by binding to the *frq* promoter and activating *frq* transcription. The WCC complex is also required for light resetting of the clock.
FRH (FRQ-interacting RNA helicase)	Forms a complex with FRQ; with FRQ acts as the negative element via repression of WCC activity. FRH is an essential gene.
VVD	A photoreceptor that is required for gating the response of the FRQ/WCC oscillator to light, entraining the clock to LD cycles, and maintaining the proper phase of the FRQ/WCC oscillator at different temperatures
CKIa	Binds and phosphorylates FRQ to promote its degradation and mediates FRQ-dependent WC phosphorylation.
CKII	Composed of CKA, CKB1, and CKB2. Essential for circadian clock function. Phosphorylates FRQ, which regulates FRQ stability and FRQ repressor activity. Mediates FRQ-dependent WC phosphorylation.
CAMK-1 (calcium-calmodulin kinase 1)	A kinase that phosphorylates FRQ in vitro. Disruption leads to changes in light-induced phase shifts and a slight change of period.
PRD4 (checkpoint kinase 2)	Phosphorylates FRQ in response to DNA damage. Not essential for clock function.
PKA	Phosphorylates FRQ to regulate its stability and functions as a priming kinase for further phosphorylation of the WCC by CKIa and CKII.
PP1	Regulates the stability of FRQ, likely through dephosphorylation.
PP2A	RGB-1, the regulatory subunit, is important for the closing of the negative-feedback loop, likely through dephosphorylating FRQ and WC proteins.
PP4	Role in dephosphorylation of the WCC, which promotes its nuclear localization.
FWD-1	An essential clock component. Interacts with FRQ and functions as the substrate-recruiting subunit of an SCF-type ubiquitin ligase that mediates FRQ ubiquitination and degradation.
CSN (COP9 signalosome)	Important for clock function by regulating the stability of the SCF^{FWD-1} complex.
CSW1	Chromatin remodeling. Loss of CSW1 affects the ability of WC-2 to leave the *frq* C-Box.
CHD2	Chromatin remodeling. Loss of CHD2 affects the ability of WC-2 to leave the *frq* C-Box.

[a]Adapted and updated from Heintzen and Liu, 2007.

et al., 1994b). Importantly, it has been demonstrated that the clock can be phase shifted through manipulation of *frq* mRNA and protein levels (Liu et al., 1998; Crosthwaite et al., 1995; Aronson et al., 1994b; Huang et al., 2006), implicating FRQ as a necessary state variable that determines both the period and the phase of the oscillation.

The full-length FRQ protein is 989 amino acids long, with a coiled-coil domain near the N terminus (Fig. 5) (Cheng et al., 2001a). FRQ dimerizes through its coiled-coil domain, and dimerization is required for its activity. Two alternatively translated forms of FRQ, large FRQ (l-FRQ) and small FRQ (s-FRQ), that differ by 99 amino acids at the N terminus are translated from two in-frame AUGs (AUG1 and AUG3) (Garceau et al., 1997; Liu et al., 1997) within different transcripts that result from alternative splicing events (Colot et al., 2005; Diernfellner et al., 2005, 2007). Low levels of FRQ are found in the nucleus, and nuclear localization is required for FRQ's role in the circadian oscillator (Luo et al., 1998). However, the vast majority of FRQ is cytoplasmic, and its function in the cytoplasm is not fully understood. Both *frq* mRNA and FRQ protein cycle with a circadian rhythm in DD, with peaks in the early morning and midday, respectively (Aronson et al., 1994b; Garceau et al., 1997). All FRQ forms a complex (FFC) with FRH, a FRQ-interacting RNA helicase (Cheng et al., 2005), and FFC functions as the negative element in the circadian negative-feedback loop. FRH is an essential protein that is a member of the SK1-2 subfamily of DEAD-box-containing RNA helicases and that plays several roles in RNA metabolism including 3′ end processing and mRNA export from the nucleus.

The WC-1 and WC-2 proteins are PAS domain-containing GATA-type zinc finger transcription factors (Ballario et al., 1996; Linden and Macino, 1997) that are found primarily in the nucleus of cells (Fig. 5) (Lee et al., 2000; Schwerdtfeger and Linden, 2000). WC-1 has three PAS domains (A, B, and C), and WC-2 has one PAS domain. The N-terminal PAS domain of WC-1 is a specialized LOV (light-, oxygen-, voltage-sensing) domain that functions as a blue-light sensory module (Liu, 2003; Briggs, 2007; Christie et al., 1999). WC-1 and WC-2 form heterodimeric complexes (the WCC complex) mediated by the PASC domain of WC-1 and the single PAS domain of WC-2 (Cheng et al., 2002, 2003; Talora et al., 1999). Both *wc-1* and *wc-2* transcripts are found at similar levels at all times of day, while curiously WC-1, but not WC-2 protein, accumulates with a circadian rhythm (He et al., 2002; Merrow et al., 2001; Lee et al., 2000).

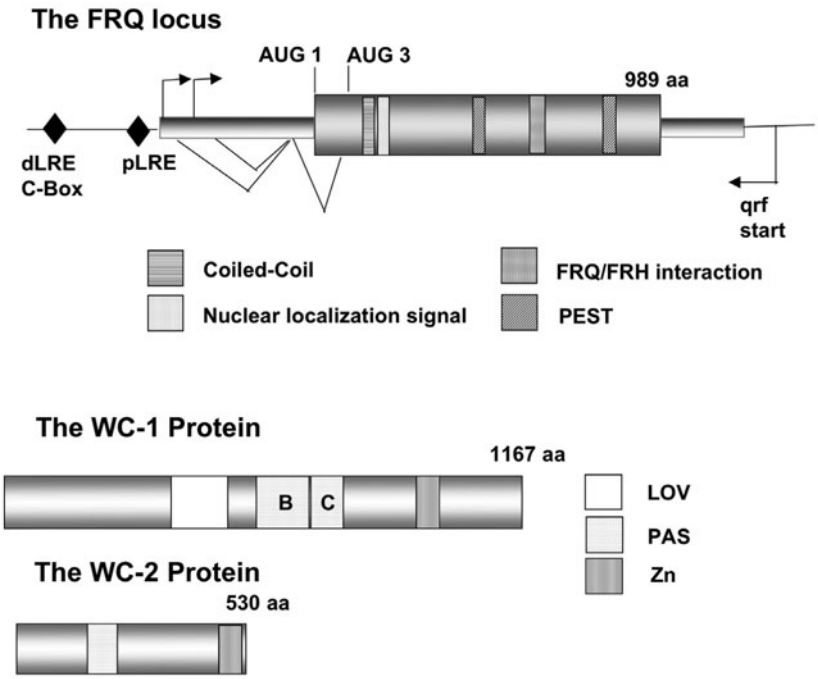

FIGURE 5 Graphic depiction of the *frq* locus and WC-1 and WC-2 proteins. See the text for details of the loci. The thin lines underneath the *frq* locus indicate alternative splicing events. Although not shown, transcription initiation of *wc-1* is initiated at several sites, and two forms of the protein are made by the use of alternative initiation sites Kaldi et al., 2006; de Paula et al., 2006).

The *Neurospora* circadian FRQ/WCC oscillator has the basic signature features of oscillators in other model systems, including *Drosophila* and mouse (Bell-Pedersen et al., 2005; Dunlap, 1999), and is depicted in Fig. 6. During late subjective night/early subjective dawn, WC-1 and WC-2 proteins heterodimerize to form the WCC. The WCC directly activates *frq* transcription by binding to specific sequences in the *frq* promoter, resulting in an increase in the levels of *frq* transcript. The promoter elements are the distal light regulator element (dLRE), also called the "Clock Box" (C-Box), that contains two GATG repeats and is required for clock and light regulation of *frq*, and the proximal light regulatory element (pLRE) required for light regulation of *frq* (He et al., 2005b; Froehlich et al., 2003). Deletion of the C-Box results in arrhythmic *frq* mRNA accumulation, indicating that binding of the WCC is essential for the function of the FRQ/WCC oscillator (Froehlich et al., 2003). Furthermore, *frq* mRNA and FRQ protein levels are very low in *wc*-null mutants; induced expression of WC proteins from an ectopic locus in *wc*-null mutants leads to rapid induction of *frq* transcription (Crosthwaite et al., 1997; Cheng et al., 2001b). Lastly, WCC that is reconstituted in vitro can bind to the C-Box in gel shift assays, indicating that the WCC does not require additional components for DNA binding (Froehlich et al., 2003; He et al., 2005b). This binding requires the Zn-finger DNA binding domains of both proteins; deletion or mutation of either Zn-finger domain abolishes the expression of *frq* in DD (Cheng et al., 2002; Collett et al., 2002; Crosthwaite et al., 1997), but the WC-1 DNA binding domain is not required for the light induction of *frq* transcription (Cheng et al., 2003). Together, these data indicate that the WCC is the primary activator of *frq* transcription.

Chromatin immunoprecipitation assays demonstrated that WC-1 is always present on the *frq* C-Box and pLRE, whereas binding of WC-2 to these sites is regulated (He et al., 2006; Belden et al., 2007). In the dark, WC-2 binding to the C-Box is rhythmic, peaking around subjective dawn, the same time of day that *frq* transcription begins (He et al., 2006). In addition, there is a low-amplitude cycle of nucleosome remodeling at the C-Box. Two ATP-dependent chromosome-remodeling enzymes were shown to be important for *frq* mRNA cycling (Belden et al., 2007). The first is a homolog of the yeast *Fun30*, mouse *Etl1*, and human SMARCAD genes, shown to be involved in chromatin remodeling, and is called *clockswitch-1* (*csw-1*) in *Neurospora*. In strains deleted for *csw-1*, *frq* mRNA is still rhythmic, but unlike in wild-type strains, the rhythm decays over time. This loss of amplitude is the result of failure of WC-2 to properly exit the C-Box, and thus, the levels of *frq* never decline to their typical baseline levels. The second component is *chd-2*, a homolog of the mammalian CHD2 and yeast *Chd1* genes. In a *Neurospora chd-2* knockout strain, *frq* mRNA levels cycle, but the overall levels are higher in the mutant than in the wild-type strains. Together these data indicate that CSW-1 and CHD-2 assist in chromatin remodeling in *Neurospora*, which in turn affects the ability of WC-2 to exit the C-Box. This regulation is important for the correct timing of *frq* expression and overt rhythmicity.

The accumulation of *frq* transcript reaches its peak around subjective midday, leading to a steady increase in the levels of FRQ protein. FRQ protein dimerizes with itself, enters the nucleus, and interacts with FRH to form the FFC (Cheng et al., 2001a, 2005). Once inside the nucleus, the FFC interacts with the WCC and promotes the phosphorylation of the WCC by casein kinase I (CKIa) and CKII (Schafmeier et al., 2005; Schwerdtfeger and Linden, 2000; Talora et al., 1999; He et al., 2005a, 2005b, 2006). Furthermore, protein kinase A (PKA) acts as a priming

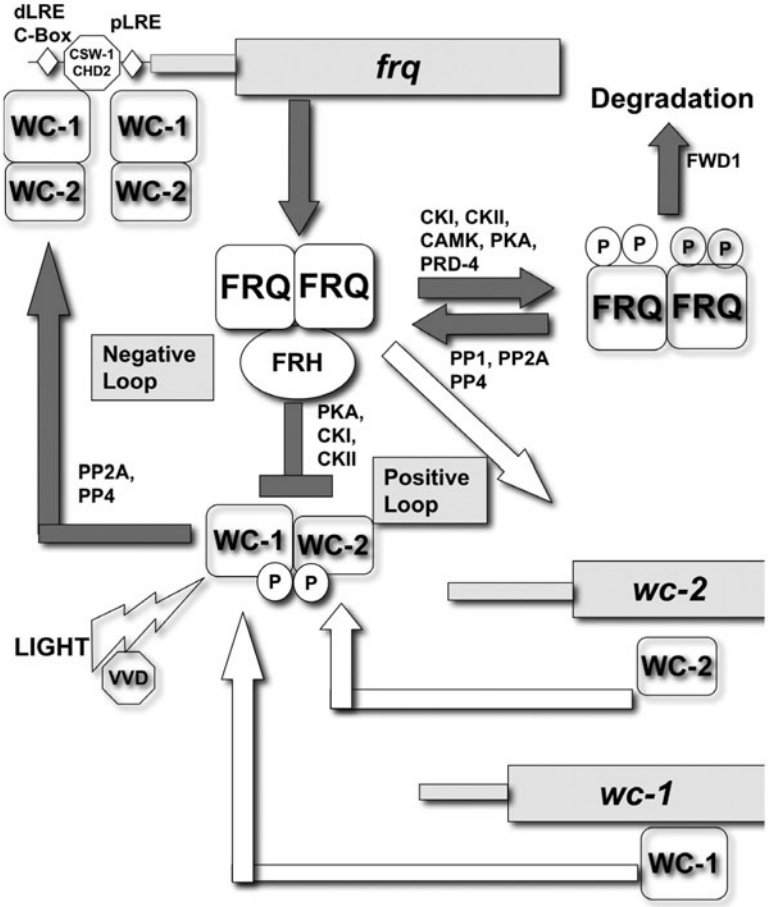

FIGURE 6 The current model of the FRQ/WCC oscillator. Both the positive loops (black arrows) and negative loops (white arrows) are shown. See the text for details of the model. P, phosphorylation.

kinase for WC phosphorylation mediated by the casein kinases (Huang et al., 2007). PKC has also been shown to phosphorylate WC-1 in vitro (Gorl et al., 2001); however, it is not clear if this can occur in vivo in the dark or whether it plays a role in the circadian feedback loop. In *frq*-null strains, both WC-1 and WC-2 are hypophosphorylated, and their normal levels of phosphorylation are restored when *frq* is expressed at an ectopic site. Furthermore, the activation of *frq* transcription at subjective dawn correlates with hypophosphorylation of the WC proteins, and a partially purified FFC complex is unable to directly inhibit the WCC binding to the C-Box in vitro. In addition, dephosphorylated WCC, but not phosphorylated WCC, binds to the C-Box (He et al., 2005b). Five major light-independent in vivo WC-1 phosphorylation sites, located immediately downstream of the WC-1 Zn-finger DNA binding domain, were identified by mass spectrometry analysis (He et al., 2005b). Mutation of these sites suggested that the phosphorylation of WC-1 negatively regulates its activity. Recently, WC phosphorylation was shown to also promote its FRQ-dependent cytoplasmic localization, thus sequestering WCC from transcriptional activation (Cha et al., 2008; Hong et al., 2008). Together these results support a model in which the FFC closes the circadian negative-feedback loop via inhibition of WCC activity by promoting the phosphorylation of the WC proteins. The net result of this inhibition is a decrease in *frq* mRNA levels (Liu et al., 2000; Schafmeier et al., 2005).

For the remainder of the day and into the early evening, the FFC presumably remains at sufficient levels to promote phosphorylation of the WCC and thus inhibit transcription of *frq*. Degradation of FRQ, in conjunction with dephosphorylation of the WCC by protein phosphatase 2A (PP2A) and PP4, eventually releases the inhibitory effect of the FFC on the WCC and leads to reactivation of *frq* transcription, allowing the cycle to start anew (Cha et al., 2008; Yang et al., 2004; Schafmeier et al., 2005; Liu et al., 2000). The dephosphorylation of WCC by PP4 promotes its nuclear localization, while PP2A may mostly activate the DNA binding activity of WCC (Cha et al., 2008).

Posttranslational Modification of FRQ Plays a Critical Role in the Function of the Oscillator Feedback Loop

In the FRQ/WCC molecular cycle, degradation and posttranslational modifications of FRQ play an essential role in period length determination and the overall function of the circadian negative-feedback loop (Liu, 2005; Pregueiro et al., 2006; Huang et al., 2007; Cheng et al., 2005; Liu et al., 2000). As soon as FRQ proteins are synthesized, they become phosphorylated. This phosphorylation is achieved by several different kinases that act sequentially. Thus, both the

amount and species of phosphorylated FRQ cycle over the course of the day. Mutations of FRQ phosphorylation sites were found to lengthen the period of the clock, suggesting that phosphorylation of FRQ promotes its turnover (Yang et al., 2003; Gorl et al., 2001; Liu et al., 2000). Five kinases, CKIa, CKII, CAMK-1 (a calcium/calmodulin-dependent kinase), PRD-4 (checkpoint kinase 2), and PKA have been identified as kinases that phosphorylate FRQ (Yang et al., 2001, 2002, 2003; Gorl et al., 2001). Disruption of the CKII catalytic subunit (*cka*) and its regulatory subunit (*ckb1*) in *Neurospora* results in high levels of hypophosphorylated FRQ and loss of normal circadian rhythmicity (Yang et al., 2002, 2003). In addition to its role in promoting FRQ degradation, CKII was shown to be important for the negative activity of FRQ in the FRQ/WCC oscillator; high levels of *frq* mRNA were observed in the *cka* mutant strain despite abundant FRQ levels. PRD-4 was identified by positional cloning of a short-period allele and functions to link the DNA damage signaling pathway to the oscillator as described later (Pregueiro et al., 2006). PKA, like the casein kinases, phosphorylates both the WCC and FRQ and acts to stabilize FRQ (Huang et al., 2007).

Neurospora CKIa is homologous to the *Drosophila* clock protein DOUBLETIME (DBT), and CKIa can phosphorylate the two PEST regions of FRQ in vitro (Gorl et al., 2001). Importantly, CKIa was found to be associated with FRQ in vivo, supporting its role as an FRQ kinase (Cheng et al., 2005; Gorl et al., 2001). Consistent with these data, deletion of the PEST-1 region of FRQ delays FRQ degradation (Schafmeier et al., 2006). CKIa is essential for cell survival in *Neurospora*. A knock-in mutant that carries a mutation equivalent to that of the *Drosophila dbt(L)* mutation established the functional importance of CKIa in the clock (He et al., 2006). In this mutant, FRQ is hypophosphorylated and is more stable, resulting in a 32-h circadian rhythm and indicating a role for CKIa in promoting FRQ degradation. In addition, both WC-1 and WC-2 are hypophosphorylated in the *ck-1a* mutant, and WCC binds to the *frq* C- box at high levels in the mutant strain. Similar results were obtained for the *cka* mutant. These data suggest that FRQ promotes the phosphorylation of WCC by recruiting the casein kinases. In support of this conclusion, mutations of FRQ that abolish the FRQ-CKIa interaction result in hypophosphorylation of WCC and arrhythmic phenotypes (He et al., 2006).

In addition to its role as a priming kinase that phosphorylates WCC, PKA is also an FRQ kinase. But unlike CKIa and CKII, which promote the degradation of FRQ, PKA stabilizes FRQ (Huang et al., 2007). In a *pkac-1* (the catalytic subunit) mutant, FRQ protein is hypophosphorylated and its levels are low in DD despite high *frq* mRNA levels. In an *mcb* (the regulatory subunit) mutant, in which PKA activity is high, *frq* mRNA levels are extremely low, but FRQ protein is at an intermediate level in DD and is more stable in the *mcb* strain. Similarly, recent studies of the Familial advanced sleep-phase syndrome mutation site in human Per2 revealed that phosphorylation of this site leads to hPer2 stabilization rather than degradation (Vanselow et al., 2006; Xu et al., 2005).

Along with the kinases, three protein phosphatases, PP1, PP2A, and PP4, are known to contribute to the control of FRQ phosphorylation (Cha et al., 2008; Yang et al., 2004). PP1 and PP4 regulate the stability of FRQ, whereas PP2A is important for the function of the negative-feedback loop. PP2A was also recently found to regulate the phosphorylation state of WC-1, a process that affects the activity of the WCC (Schafmeier et al., 2005).

FWD-1, an F-box WD-40 repeat-containing protein and the *Neurospora* homolog of the *Drosophila* protein Slimb, mediates the phosphorylation-dependent degradation of FRQ (Grima et al., 2002; He et al., 2003). FWD-1 physically interacts with phosphorylated forms of FRQ and serves as the substrate-recruiting subunit of an SCF-type ubiquitin ligase (E3) to mediate FRQ ubiquitination. In an *fwd-1* mutant strain, circadian rhythms are abolished and FRQ protein accumulates to high levels in its hyperphosphorylated state. In addition, the COP9 signalosome (CSN), a conserved multisubunit complex in all eukaryotes, was shown to be important for clock function by regulating the stability of the SCF^{FWD-1} complex in *Neurospora* (He et al., 2005a).

Together, these data suggest that the progressive phosphorylation of FRQ, regulated by multiple kinases and phosphatases at several independent sites, fine-tunes the stability of FRQ and is a major player in delaying the molecular feedback loop and thus in determining the period length of the clock.

Interconnected Negative- and Positive-Feedback Loops

While the main function of FRQ in the circadian oscillator is its action to negatively regulate its own expression, FRQ also functions to promote the expression of WC-1 and WC-2, forming positive-feedback loops that are interlocked with the negative loop (Merrow et al., 2001; Cheng et al., 2001b, 2003; Lee et al., 2000). FRQ regulates WC-1 expression posttranscriptionally, while it appears to promote *wc-2* transcription initiation (Schafmeier et al., 2006; Cheng et al., 2001b; Lee et al., 2000). Although the mechanism(s) by which FRQ carries out these functions has not been fully elucidated, it was shown that phosphorylation of the PEST-2 region of cytoplasmic FRQ is required to promote WC-1 accumulation (Schafmeier et al., 2006). Furthermore, mutation of the putative FRQ phosphorylation sites in the PEST2 region results in arrhythmic conidiation. Experiments in which *wc-1* or *wc-2* was overexpressed to different levels from an inducible promoter demonstrated that while rhythmic WC-1 is dispensable for overt rhythmicity, the positive-feedback loops are important for maintaining the amplitude and precision of the developmental rhythm (Cheng et al., 2001b), supporting a role for the positive feedback in clock function (Schafmeier et al., 2006). However, additional mechanisms must contribute to the WC-1 accumulation rhythm since this rhythm is observed in FRQ-null strains when cultures are synchronized by a temperature transition (de Paula et al., 2006).

WC-1 and WC-2 also regulate each other to form an additional interacting feedback loop. The levels of WC-1 protein are very low in a *wc-2* deletion strain or in strains in which the WC-1/WC-2 interaction is disrupted, indicating that WC-2 is required for maintaining WC-1 levels in the cell through formation of the WCC (Cheng et al., 2002). In other words, WC-1 protein is unstable by itself. WC-1 also negatively regulates *wc-2* expression, either directly or indirectly, at the level of transcript abundance (Cheng et al., 2003). While the details of these regulations are not understood, the additional feedback loops are thought to contribute to maintaining optimal levels of the WCC in the cell for its role in the clock and in blue-light responses (see below) (Heintzen and Liu, 2007).

The net result of all the various activities of FRQ described above is a stable and robust circadian cycle in which early events lead to negative feedback. Late actions promote the appearance of WC-1 and WC-2 such that phosphorylated WCC is maintained at a high level but in an

inactive state. Eventually, the phosphorylation-mediated turnover of FRQ releases the phosphorylated WCC. Through the action of protein phosphatases that act upon phosphorylated WCC, or through new synthesis and assembly of the WCC, transcription of *frq* is initiated in the subsequent daily cycle. The long period of this cycle (22.5 h in DD) can be explained in large part by the amount of time it takes for FRQ to be phosphorylated and turned over (Merrow et al., 1997). This delay is based on the action of several different kinases that are thought to work sequentially such that they can delay each subsequent phosphorylation event (Huang et al., 2007).

CONSERVATION OF EUKARYOTIC CIRCADIAN OSCILLATORS

Despite the evolutionary distance between *Neurospora* and higher eukaryotes, remarkable conservation exists between the circadian oscillators of *Neurospora* and those of *Drosophila* and mammals. This conservation is observed at the level of gene network organization, gene regulation, and component structure.

As mentioned earlier, the circadian oscillators of *Neurospora*, *Drosophila*, and mammals that have been described are all based on autoregulatory negative-feedback loops (Young and Kay, 2001; Dunlap, 1999). All of these representative circadian negative-feedback loops arise from interactions between positive and negative elements. The positive elements are all heterodimeric complexes consisting of two PAS domain-containing transcription factors: WC-1 and WC-2 in *Neurospora*, dCLOCK and CYCLE in *Drosophila*, and CLOCK/NPAS2 and BMAL1 in mammals. In each case, the positive elements activate the transcription of the negative elements, likely by directly binding to the promoters of the genes encoding the negative elements. The negative elements (FFC in *Neurospora*, PERIOD [PER] and TIMELESS in *Drosophila*, and PER and CRYPTOCHROME in mammals) inhibit the activity of the positive elements through physical interactions and by recruiting kinases (Kim and Edery, 2006; Yu et al., 2006). In addition to the negative-feedback loop, interlocked positive-feedback loops are found in all three systems, and they share a role in promoting the robustness and stability of the clock (Preitner et al., 2002; Kim et al., 2002; Cheng et al., 2001b; Lee et al., 2000; Shearman et al., 2000; Glossop et al., 1999).

Similar to FRQ and the WC proteins, the core clock proteins in *Drosophila* and mammals are posttranslationally regulated by phosphorylation, which ultimately triggers their ubiquitination and proteasome-mediated degradation (He et al., 2003; Eide et al., 2005; Ko et al., 2002; Grima et al., 2002; Young and Kay, 2001). In addition, the phosphorylation state of *Drosophila* CLOCK, like *Neurospora* WC proteins, exhibits a circadian rhythm and its phosphorylation is dependent on PER (Yu et al., 2006; Kim and Edery, 2006). Furthermore, an RNA binding protein is associated with PER in mammals and *Drosophila*, suggesting that the regulation of RNA metabolism may be another conserved aspect of circadian oscillators (Brown et al., 2005).

As previously mentioned, WC-1 protein contains 3 PAS domains. The N-terminal PAS domain (PAS A) is a specialized LOV domain that functions as the blue-light sensing module. The LOV domain is a conserved domain found also in plant phototropins, including the circadian clock photoreceptor ZEITLUPE (Kim et al., 2007; Cheng et al., 2003). The other PAS domains (PASB and PASC) show similarity to PAS domains in the mammalian and fly positive clock components BMAL1 and CYC (Tauber et al., 2004; Lee et al., 2000). Aside from these similarities, there is little sequence conservation between key clock components in *Neurospora* and those of *Drosophila* and mammals. Yet the regulatory components that are critical for posttranslational regulation of the clock components are highly conserved from *Neurospora* to mammals. FRQ and PER, while not sequence homologs, are phosphorylated by the same kinases and dephosphorylated by the same phosphatases (Kloss et al., 1998; Yang et al., 2002, 2003, 2004; Gorl et al., 2001; Young and Kay, 2001; Price et al., 1998; Lowrey et al., 2000; Xu et al., 2005; Akten et al., 2003; Lin et al, 2002; Nawathean and Rosbash, 2004; Sathyanarayanan et al., 2004; Huang et al., 2007; Matsuoka et al., 2007). In addition, phosphorylation of FRQ and PER promotes their degradation, and phosphorylation is important for their repressor activities. The phosphorylation-dependent ubiquitination and degradation of PER and FRQ are mediated by a conserved SCF E3 ligase, with FWD-1 homologs as the substrate-recruiting subunits (He et al., 2003; Eide et al., 2005; Ko et al., 2002; Grima et al., 2002).

The conservation observed among the eukaryotic circadian oscillators further validates the use of *Neurospora* as an instructive model system for eukaryotic circadian systems, including the human clock mechanism.

INPUT PATHWAYS ALLOW FOR ENTRAINMENT AND RESETTING OF CIRCADIAN OSCILLATORS

The FRQ/WCC oscillator components form the core of the biological clock responsible for rhythms that persist in the absence of environmental cues. However, to stay in synchrony with the natural world, circadian clocks must be coupled to the 24-h environmental rhythms. This coupling process is called entrainment. An entrained circadian system produces rhythms that occur at their optimal time each day and throughout the yearly changes of the seasons. Not surprisingly, among all of the environmental factors, light and temperature are two of the most important inputs for entrainment of circadian clocks. Currently, *Neurospora* is the best-understood circadian system in terms of environmental inputs (Liu, 2003). In addition, *Neurospora* has served as an important model system for the understanding of light responses in fungi.

WC-1 Is the Blue-Light Photoreceptor for the Circadian Clock and Other Light Responses

Almost all known *Neurospora* light responses, including light resetting of the clock, are mediated by blue light (Linden and Macino, 1997). In addition to the role of WC-1 and WC-2 in the circadian feedback loops, these two transcription factors are essential for *Neurospora* blue-light responses (Ballario et al., 1996; Linden and Macino, 1997; Liu, 2003). WC-1 is the main blue-light receptor in *Neurospora*, further demonstrating the link between light input and the circadian clock. The LOV domain of WC-1 binds the flavin chromophore FAD (flavin adenine dinucleotide) (Froehlich et al., 2002; He et al., 2002). Purified WC complexes from *Neurospora*, or from a heterologous expression system, are associated with FAD, and mutations of the WC-1 flavin-binding site abolish its light functions (Cheng et al., 2003; He et al., 2002, 2005b). Based on studies of LOV domains in plant and fungal photoreceptors (Crosson et al., 2003; Zoltowski and Crane, 2008), binding of FAD to WC-1 likely results in a conformational change in WC-1, which may open up target sites for kinases and phosphatases. In support of this model, both WC-1 and

WC-2 undergo light-induced changes in phosphorylation status (Schwerdtfeger and Linden, 2000; Talora et al., 1999). For example, WC-1 becomes hyperphosphorylated following light exposure but quickly returns to the hypophosphorylated state during extended periods of illumination (Schwerdtfeger and Linden, 2000; Talora et al., 1999; He et al., 2005b; Heintzen et al., 2001). Light-induced changes in phosphorylation of WC-1 may in turn lead to a change in the ability of WC-1 to interact with WC-2, a change in WC-1 interaction with downstream signaling components, or a change in the stability of WC-1. Consistent with this idea, several studies have suggested that WC-1 phosphorylation in response to light triggers its degradation (Talora et al., 1999; Lee et al., 2000; He et al., 2005b). Similar to other photoreceptors, WC-1 has a photocycle in vivo, but the half-life of its photocycle is very long (>1 h), suggesting that WC-1 cannot be efficiently used for repeated photoactivation (He et al., 2005b). WC-2 is also required for all light responses; however, the role of WC-2 in light responses is not as a photoreceptor; instead, it probably functions in light responses through its ability to stabilize WC-1 (Liu, 2003).

How Light Resets the Clock and Triggers Light-Induced Transcription

In order for entrainment to work, light must delay the clock into the previous day when detected in the early night, and advance the clock into the next day when detected at late night. In other words, the same light signal must have opposing effects on the timing mechanisms depending on when during the circadian cycle light is perceived (Crosthwaite et al., 1995). The discovery that light rapidly induces transcription of *frq* provided a molecular explanation for light-induced clock-resetting behavior in eukaryotes: a light-induced change in the levels of a key oscillator component (in this case, *frq* mRNA) leads to a change in the position (phase shift) of the clock (Fig. 7) (Crosthwaite et al., 1995). Light treatment during the subjective evening, when *frq* mRNA levels are falling, results in *frq* induction. The resulting high levels of *frq* mRNA would send the

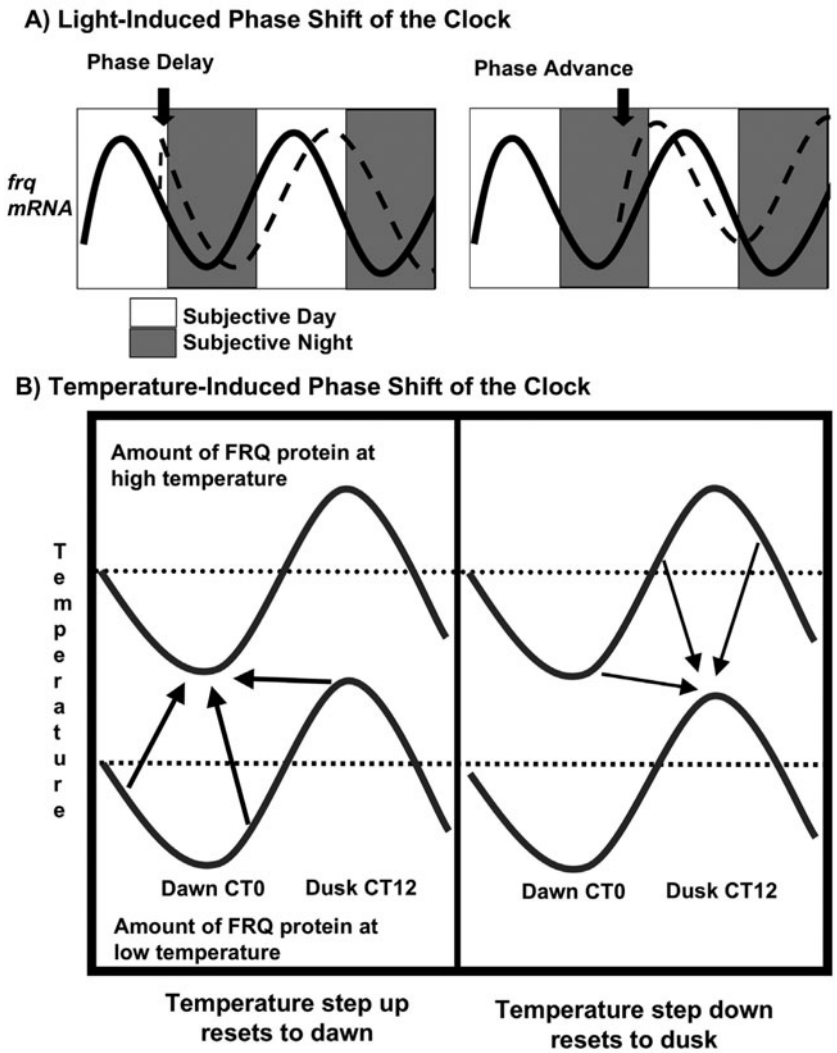

FIGURE 7 How light (A) and temperature (B) reset the *Neurospora* circadian clock. See the text for details of the models.

clock back toward the time of day when *frq* mRNA levels peak (around midday), causing a phase delay of the oscillator. On the other hand, light treatment in the subjective late night, when *frq* levels are rising, results in an instantaneous induction of *frq* and advances the clock to the midday peak. Light treatment at midday, when *frq* levels are already at their peak, has little or no effect on the clock.

Subsequently it was shown that the *wc* genes are required for light induction of *frq*, demonstrating an essential role for the WC proteins in light input to the clock (Crosthwaite et al., 1997). After light exposure, at any time of day, a large WC complex (L-WCC) is activated through the WC-1 photosensory LOV domain (Froehlich et al., 2002). The L-WCC consists of only WC-1 and WC-2 but has more than one WC-1 molecule (Cheng et al., 2003; He et al., 2005b; Froehlich et al., 2002). This light-activated L-WCC then binds to the *frq* promoter C-Box and pLRE (Fig. 5), leading to the activation of *frq* transcription (He et al., 2005b; Froehlich et al., 2002). The light-induced transcriptional activation of *al-3* and *vvd*, two other *Neurospora* immediate light-inducible genes, was shown to use a mechanism similar to that of *frq*, suggesting a common molecular basis for light responses in *Neurospora* (He et al., 2005b). WC proteins become hyperphosphorylated after light exposure (Schwerdtfeger and Linden, 2000; Talora et al., 1999), and similar to its function in the dark, light-induced WC phosphorylation inhibits L-WCC DNA binding activity and promotes its degradation (He et al., 2005b; Lee et al., 2000). As a result, L-WCC binds to the LREs only transiently. This transient response to light underlies the mechanism of photoadaptation, a process whereby light-regulated processes are downregulated under continuous illumination (Heintzen and Liu, 2007; He and Liu, 2005a; Schwerdtfeger and Linden, 2000, 2003).

An antisense *frq* transcript that is not predicted to encode any protein, called *qrf*, influences light entrainment of the *Neurospora* clock (Kramer and Crosthwaite, 2007; Kramer et al., 2003). The *qrf* transcript cycles in abundance in the opposite phase of *frq*, and its levels are strongly light induced. Elimination of this transcript resulted in a stronger resetting response to light pulses. In strains that lack *qrf*, the circadian clock functions normally in DD. These data suggest that *qrf* mutes the light response of the clock. However, the mechanism for *qrf* function is unknown.

Photoperiodic Responses in *Neurospora* and the Role of VVD in Clock Entrainment

Rapid changes in the levels of oscillator components (such as FRQ in *N. crassa)* provide an explanation for clock resetting by brief light pulses; however, they do not explain how the organism deals with continuous light during the day. It has been suggested that dawn and dusk transitions are the only changes that are sensed by the clock and that a clock-modulated light response can block the action of light during the day (Johnson et al., 2003). This time-of-day-dependent modulated response to an environmental stimulus is a process called gating. In *Neurospora*, gating requires an additional feedback loop involving VIVID (VVD). VVD is a small LOV/PAS domain-containing protein that noncovalently binds a FAD that serves as a chromophore. When the FAD absorbs blue light, the light-induced cysteinyl adduct formed between the chromophore and VVD triggers conformational changes in the N-terminal helix of VVD and subsequent VVD homodimerization (Zoltowski and Crane, 2008). The structural changes associated with VVD homodimerization (or possibly heterodimerization with other LOV domain-containing proteins, such as WC-1) may trigger the signaling events that allow VVD to modulate light signaling pathways (Heintzen et al., 2001; Schwerdtfeger and Linden, 2003; Shrode et al., 2001; Zoltowski and Crane, 2008; Cheng et al., 2003). In *vvd* mutants, WCC-dependent blue-light responses are elevated and photoadaptation is partially lost, indicating that VVD functions as a general repressor of light responses. *vvd* transcripts are also clock controlled. Clock regulation of *vvd*, together with the action of VVD as a repressor of light responses, fulfills the requirements for a molecular gate; the clock would control its own light sensitivity by rhythmically regulating VVD levels. This idea is consistent with the observations that while VVD is not essential for the circadian clock to run in DD, *vvd* mutants exhibit an increased resetting response to light pulses and a diminished, but not abolished, ability to gate the response to light (Heintzen et al., 2001).

Recently it was shown that VVD also plays a role in entraining the clock to different photoperiods. Under LD cycles, VVD reduces light resetting at dawn, probably by inhibiting WCC activity, while it promotes resetting of the clock at dusk by accelerating the decrease of *frq* RNA levels (Elvin et al., 2005). Thus, VVD may help to entrain the *Neurospora* circadian clock properly under natural photoperiods. Finally, VVD also has a role in ensuring that the phase of the overt rhythms is fairly constant across a range of temperatures (Hunt et al., 2007). As suggested above, the mechanism by which VVD exerts its functions is likely to involve direct or indirect effects on WCC phosphorylation and activity. WC-1 is constitutively hyperphosphorylated in *vvd* mutant strains, suggesting that VVD activates a phosphatase or represses a kinase. However, because hyperphosphorylated WC-1 is the inactive form of WC-1, one would anticipate a decreased light response, rather than the observed increased response to light in the *vvd* mutants. Therefore, the regulation of light signaling must be more complex, likely involving other factors or activities for activation and transitional states of light responses in the organism (Heintzen and Liu, 2007).

Photoperiodic responses allow organisms to adapt to all seasons, and one role for circadian clocks is to provide organisms with the ability to measure changes in day length. Photoperiodic production of sexual spores, asexual spores, and synthesis of carotenoids was recently observed in *Neurospora* (Tan et al., 2004). By systematically varying the day length, it was found that the production of conidiospores peaked around equinox and protoperithecia and carotenoids peaked in long days. These photoperiodic responses were abolished in strains that lacked FRQ, indicating that seasonal responses require a functional FRQ/WCC oscillator. At the molecular level, it was demonstrated that under different photoperiods, the phase of *frq* RNA is phase locked by the light-to-dark transitions, while the phase of FRQ protein is not. These data suggested the existence of a day-length-dependent posttranscriptional mechanism that regulates the levels of FRQ (Tan et al., 2004).

Other Light Input Mechanisms

Mining the *Neurospora* genome sequence revealed additional light-signaling proteins with similarities to phytochromes (PHY-1 and PHY-2), cryptochrome (CRY), opsin (NOP-1), and LOV domain-containing proteins (PHOT) (Borkovich et al., 2004; Froehlich et al., 2005). These findings suggested that other blue- or red-light signaling pathways exist in *Neurospora*. CRY expression is light regulated, and the clock regulates *phy-1* transcription

(Froehlich et al., 2005). However, gene knockouts (single or in combinations) have not helped to determine their function (Heintzen and Liu, 2007). Thus, the possible role of these proteins in the clock has remained elusive.

Temperature Effects on the Clock

Temperature affects the clock in three ways. First, all clocks entrain to temperature steps and cycles, with temperature steps resetting the oscillator in a manner similar to light pulses (Francis and Sargent, 1979). Second, there are physiological limits in temperature that permit the normal operation of the clock. Third, the period of the clock is temperature compensated. Similar to light entrainment, the mechanism of temperature effects on the clock appears to involve changes in the amount and perhaps the form of FRQ protein that is made. However, unlike light input, temperature-mediated effects do not appear to involve an independent temperature sensor molecule (Liu et al., 1998).

Temperature Resetting

Clock resetting by temperature steps involves posttranscriptional regulation of FRQ. *frq* transcript levels are not greatly affected by temperature; however, as the temperature increases, the amount of FRQ protein increases (Colot et al., 2005; Diernfellner et al., 2005; Liu et al., 1998). As a result, FRQ levels oscillate at a higher mean at higher temperatures. In other words, oscillations with peak levels of FRQ in the late day to early evening and troughs in the late night continue at high temperature, but the number of FRQ molecules associated with a particular time of day is different at different temperatures. Upon a temperature step-up, the current level of FRQ protein becomes the new trough level (equivalent to dawn) of a phase-shifted cycle; upon a temperature step-down, the current level of FRQ becomes the new peak level (equivalent to dusk) of a phase-shifted cycle, thus resetting the clock to a new phase in both instances (Fig. 7) (Liu et al., 1998). The general increase in FRQ levels at high temperature is not controlled by temperature-sensitive splicing events (see below) but instead is at least partly due to a temperature-dependent translation control mechanism mediated by upstream open reading frames in the *frq* 5′ untranslated region (Diernfellner et al., 2005). The upstream open reading frames are efficiently translated at low temperature as a result of their poor translational context, and their translation reduces the translation efficiency of l-FRQ and s-FRQ.

Physiological Limits for Circadian Rhythmicity

In *Neurospora*, the range of temperatures over which the clock functions properly is between 18 and 30°C (Francis and Sargent, 1979), and this limit is regulated by the ratio of two forms of FRQ protein in the cell (Liu et al., 1997). Thus, in addition to changes in the absolute levels of FRQ, temperature also affects the ratio of these forms. It is thought that the correct ratio between the two forms is necessary to support rhythms over a broad range of temperatures: strains engineered to express only s-FRQ are rhythmic at low but not high temperatures, and strains that express only l-FRQ are rhythmic at high but not low temperatures (Liu et al., 1997). The same *frq* transcript encodes both forms of FRQ protein, and temperature-sensitive alternative splicing events determine the relative levels of each form (Fig. 5) (Colot et al., 2005; Diernfellner et al., 2005). The start codon of l-FRQ is contained within an intron with nonconsensus splice sites. At lower temperatures, this intron is spliced much more efficiently than at elevated temperature. Thus, at low temperature, translation occurs primarily from the second, downstream start site (AUG3) producing s-FRQ and reduces the production of l-FRQ. At higher temperatures, the recognition of the nonconsensus splice sites of the intron is much less efficient, which leaves the l-FRQ start codon in place (AUG1), and increased production of l-FRQ occurs. The end result of these alternative splicing events is that the ratio of l-FRQ to s-FRQ increases with an increase in temperature, conferring rhythmic behavior over a broad range of temperatures. Interestingly, the clock can function with either form of FRQ if there is enough of it (Colot et al., 2005; Diernfellner et al., 2005), and there is no difference in the stability of the two forms of FRQ. Circadian rhythms in strains that express only l-FRQ or s-FRQ are temperature compensated (Diernfellner et al., 2007), indicating that the basis of temperature compensation does not lie in the regulation of the relative levels of the two forms of FRQ protein. Instead, the temperature-dependent synthesis of l-FRQ and s-FRQ may allow fine-tuning of the period or adjust the *frq* mRNA oscillation in response to changes in ambient temperature (Diernfellner et al., 2007).

Consistent with the role of FRQ as a temperature sensor, a limited microarray study identified 14 genes that responded to a 12-h temperature cycle (Nowrousian et al., 2003). Each of the 14 genes was shown to be clock controlled and required FRQ protein for rhythmicity. In *frq*-null strains, the temperature response of the genes was also lost. In that study no genes that responded to temperature in the *frq*-null strain were found. While these data are consistent with FRQ acting as a temperature sensor, the loss of a temperature response could also be explained by the absence of a functional FRQ/WCC oscillator. The inability to identify genes in the microarray study that respond to temperature in the FRQ-null strain might also suggest that most temperature responses are the result of posttranscriptional regulation. Lastly, while the data are consistent with FRQ functioning as a temperature sensor for *Neurospora* cells, temperature responses can be observed in FRQ-less strains (Merrow et al., 1999; de Paula et al., 2006). Thus, FRQ cannot be the only component in *Neurospora* that mediates temperature responses.

Temperature Compensation of the Clock

Several period mutants in *Neurospora*, as well as other organisms, are associated with a partial loss of temperature compensation. This may be due to secondary effects, such as temperature-sensitive mutations in clock proteins that affect their stability and activity. Indeed, secondary effects are observed in the *Neurospora prd-4* mutation. PRD-4 functions as the DNA-damage-activated protein checkpoint kinase 2. PRD-4 can phosphorylate FRQ in response to DNA damage and destabilize the protein (Pregueiro et al., 2006). A mutation that causes PRD-4 to become active, even in the absence of DNA damage, results in temperature-dependent stabilization of FRQ and a partial loss of temperature compensation. On the other hand, the study of two other mutations that result in enhanced compensation of the clock were more informative with regards to the mechanism of temperature compensation. In wild-type strains, the *Neurospora* clock is slightly undercompensated; the period gets shorter as the temperature increases, up to about 30°C. Beyond 30°C, compensation is lost. In *prd-3* and *chr* mutant strains, the rhythms are overcompensated: the period increases as the temperature increases (Feldman et al., 1979). These genes encode separate subunits of the

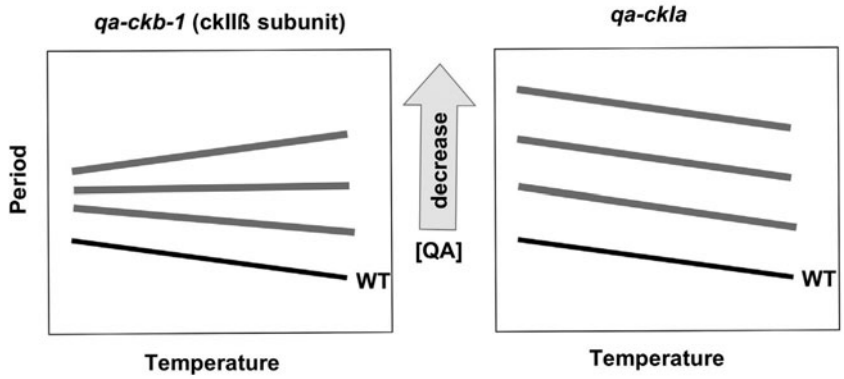

FIGURE 8 The role of CKIIβ in temperature compensation of the *Neurospora* clock. Induced expression of *ckb-1* (the β subunit of CKII) from a quinic acid-inducible promoter (*qa*) affects temperature compensation of the clock. As the concentration of quinic acid (QA) decreases, the levels of CKII decrease. This results in changes in temperature compensation of the developmental rhythm from slightly undercompensated, similar to the wild type (WT; black line), to overcompensation. In contrast, as the levels of CK1a drop, the period changes, but temperature compensation is still slightly undercompensated at all levels of CK1a.

same holoenzyme, CKII (Dunlap et al., 2007). Strains in which the levels of CKIIα or CKIIβ could be manipulated demonstrated that between 18 and 30°C, high levels of CKIIβ result in slight undercompensation, similar to what is observed in wild-type strains. As the dosage drops, compensation gets better than normal, and as the dosage drops further, strains became overcompensated (Fig. 8). Importantly, not every kinase that phosphorylates FRQ affects temperature compensation. For example, manipulation of the levels of CKIa affects period, but the temperature compensation profile is not affected in the mutant strain. While a molecular explanation for the role of CKII in temperature compensation of the clock is lacking, these data support the idea that CKII regulates the pace of the clock at different temperatures through FRQ modification.

OSCILLATOR COMPLEXITY

The more we continue to probe circadian systems, especially under different environmental conditions, the clearer it becomes that the circadian system constitutes a network of oscillators. Several lines of evidence point to the existence of multiple oscillators in cells and/or tissues of organisms contributing to circadian timing. First, free-running rhythms with different period lengths can exist in the same organism (Cambras et al., 2007; Sai and Johnson, 1999; Morse et al., 1994), and there is residual rhythmicity in strains that are defective in known oscillator components, including *Neurospora* (Collins et al., 2005; Emery et al., 2000; Stanewsky et al., 1998; Loros and Feldman, 1986). Second, some tissue-specific oscillators in higher eukaryotes are constructed differently from the pacemakers located in the brain (Ivanchenko et al., 2001; Stanewsky et al., 1998; Collins et al., 2005; Hardin et al., 2003; Krishnan et al., 2001). There are several reasons to suspect that organisms would benefit from having multiple oscillators. First, the multiple oscillators might have evolved to allow the circadian system to be more robust (resistant to stochastic noise or to small perturbations). This is supported by the observation that alteration of the levels of components of the loops results in less precise clocks (de Paula et al., 2007; Preitner et al., 2002; Locke et al., 2006; Cheng et al., 2001b). Second, multiple coupled oscillators could add to the degree of flexibility of the circadian network by providing a mechanism for the environment to entrain the different loops with different input signals, so that there may be one oscillator that is reset by light input and a different oscillator that is reset by temperature (Rand et al., 2006). Third, increased flexibility of the system could be achieved using multiple oscillators that respond differently to environmental input and regulate different phase-specific outputs; for example, morning-specific outputs would be controlled by an oscillator with one set of light and temperature inputs, while evening-specific outputs would be controlled by a different oscillator with a different set of light and temperature inputs.

In *Neurospora*, evidence for the existence of multiple oscillators first arose with observations of residual developmental rhythms in strains that lack FRQ, WC-1, or WC-2 in DD (Lakin-Thomas, 2006; Aronson et al., 1994a; Merrow et al., 1999; Loros and Feldman, 1986; Pregueiro et al., 2005; Lakin-Thomas and Brody, 2000; Lakin-Thomas, 1998; Mattern et al., 1982; Mattern and Brody, 1979; He et al., 2005a). However, these residual rhythms in development are defective in one or more of the canonical circadian oscillator properties: the rhythms are not stable, do not persist under constant conditions, are not temperature compensated, and do not entrain to LD cycles. These data suggested that an intact FRQ/WCC oscillator is required for complete circadian properties of the developmental rhythm. However, the developmental rhythm can be synchronized by temperature cycles in the absence of FRQ (Merrow et al., 1999). Some interpret the FRQ-less developmental rhythms in temperature cycles as evidence for circadian entrainment (Roenneberg et al., 2005; Lakin-Thomas, 2006; Merrow et al., 1999), whereas others view it as a temperature-driven process that terminates in the absence of external temperature cycles (Pregueiro et al., 2005). This controversy is centered on the nature of the temperature-controlled FRQ-less oscillations. Is the FRQ-less oscillation in development derived from a circadian oscillator that can be entrained, or rather from a noncircadian

oscillator that is driven by environmental signals? Full resolution of this controversy will require the identification of the molecular components of the FRQ-less oscillator (FLO) and a study of mutations in these components.

Other support for the existence of FLOs in *Neurospora* arose from the observation that in cultures grown in media with nitrate as the sole nitrogen source, rhythms in nitrate reductase enzymatic activity persist in DD and LL in wild-type strains and in strains that lack FRQ or WC-1 (Christensen et al., 2004). These data indicated that a nitrate reductase oscillator, which can run in the absence of a functional FRQ/WCC oscillator, generates rhythms in nitrate reductase activity. Yet in the absence of WC-1, the nitrate reductase activity rhythms are of low amplitude and more variable, suggesting some connection to the FRQ/WCC oscillator, or possibly even a different oscillator loop that requires WC-1. It is not yet known if the nitrate reductase oscillator is temperature compensated or entrained by light or temperature.

Evidence exists for an additional oscillator that shares components with the FRQ/WCC oscillator but that regulates distinct outputs. This WC-dependent FLO (called the WC-FLO) is responsible for generating temperature-responsive rhythms in mRNA accumulation of the evening-specific *clock-controlled gene-16* (*ccg-16*) gene (de Paula et al., 2006) (see below for a full description of ccgs). Circadian rhythms in *ccg-16* mRNA accumulation persist in the absence of FRQ and thus of a functional FRQ/WCC oscillator, indicating the autonomous nature of the WC-FLO. Northern assays with RNA isolated from liquid shaking cultures (see Fig. 9 for an example) grown at three different temperatures and harvested every 4 h over 2 days were initially used to test if *ccg-16* rhythms are temperature compensated in the absence of FRQ (de Paula et al., 2006). The results were consistent with temperature compensation; however, because the interval between the time points was 4 h, differences in period could have been missed. Thus, these experiments need to be verified using a shorter sampling interval. This can now be accomplished by continuous monitoring of living cells harboring a *ccg-16*-promoter:luciferase reporter fusion (see below) (Fig. 10). Although the rhythms in *ccg-16* mRNA levels are independent of FRQ protein, they do require WC-1 and WC-2 proteins. These results suggested the possibility that the FRQ/WCC oscillator and the WC-FLO are coupled either directly or indirectly through the WC proteins.

These observations support the hypothesis that the cellular circadian clock of *Neurospora* is a network composed of coupled oscillators: the circadian FRQ/WCC oscillator and the FLOs. The presence of multiple oscillators within these cells may contribute to the diversity of rhythmic processes under clock control, such as conidiation versus expression of genes unrelated to development. An alternative view is that the FRQ/WCC oscillator is a pacemaker that receives light and temperature input from the environment and sets the pace of one or more slave

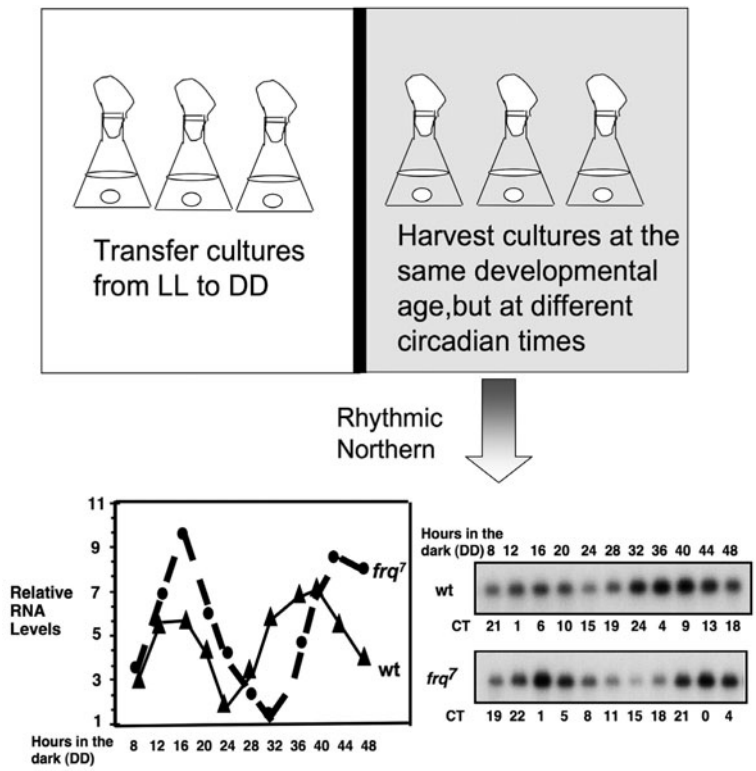

FIGURE 9 Rhythmic liquid culture assay. To isolate RNA or protein from cultures harvested at different times of day, liquid shaking cultures are routinely used (Loros et al., 1989). Transfer of the cultures from LL to DD is staggered to allow harvesting at different circadian times but near the same developmental age. An example of a rhythmic Northern blot is shown for the morning peaking clock-controlled gene *ccg-2* from a wild-type (wt) and *frq*^{7} long-period (29-h) mutant strain. Note that at DD32, *ccg-2* mRNA cycles almost 180° out of phase in the mutant versus the wt strain, reflecting the long period of the *frq*^{7} allele.

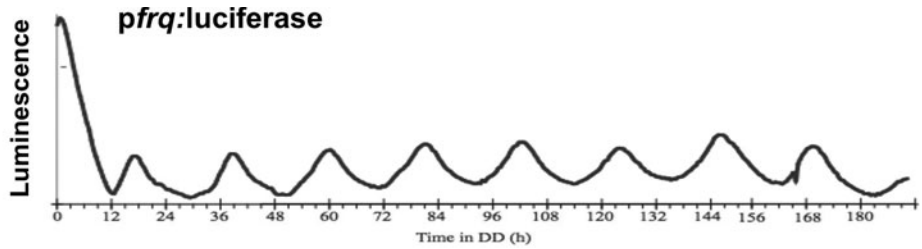

FIGURE 10 Rhythmic luciferase assays. By using a codon-optimized firefly luciferase, rhythmicity can be monitored by generating promoter fusions to the modified luciferase open reading frame (Gooch et al., 2008; Morgan et al., 2003). Luciferase activity is determined by the level of luminescence, as shown here for the *frq* promoter (p):luciferase fusion expressed in *Neurospora* cells (Shi et al., 2007).

oscillators (the FLOs) that require the FRQ/WCC oscillator for full circadian properties. A similar hierarchical organization of oscillators exists in mammals. However, unlike in *Neurospora*, in which multiple oscillators composed of distinct molecular components are present within a single cell, the clock in mammals is composed of an oscillator network that includes a pacemaker present in one tissue (the brain) that coordinates similar molecular oscillators present in the cells of different tissues (such as the liver) (Bell-Pedersen et al., 2005).

To date, none of the molecular constituents of the FLO(s) responsible for the residual developmental rhythmicty or the WC-FLO have been identified. Progress in identifying FLO components involved in the developmental rhythm has been hampered by the variability of the rhythm, making it difficult to screen for mutants that disrupt the FLO oscillations. However, recently it was shown that the period of the FLO conidiation rhythm can be stabilized, through an unknown mechanism, by the addition of farnesol or gerianiol, two intermediates of the sterol synthesis pathway, to the growth medium (Granshaw et al., 2003). Similar to the variable rhythm on unsupplemented medium, this stabilized rhythm still lacks some of the canonical properties of circadian oscillators, including temperature compensation and light entrainment, but the rhythm can be reset by temperature pulses. The ability to now observe stable developmental rhythms in the absence of a functional FRQ/WCC oscillator will facilitate the application of genetic screens for mutations that alter the FLO (Lombardi et al., 2007). Furthermore, the identification of molecular rhythms in *ccg-16* under control of the WC-FLO provides a route to identifying components of this oscillator by screening for mutant strains that alter or abolish *ccg-16* mRNA rhythms by using a *ccg-16* promoter-driven luciferase reporter system (Gooch et al., 2008; Morgan et al., 2003).

One recent study has begun the process of dissecting the network of oscillators to discern which might be a part of the oscillatory system and which might be a part of the output or not connected in any way to the circadian system. This question is more difficult than it seems at first because often the alternative oscillators are observed only in the absence of the FRQ/WCC feedback loop. One of these is a choline deficiency oscillator (CDO), observed in *chol-1* mutants grown under choline starvation, that drives an uncompensated long-period developmental cycle (approximately 60 to 120 h under full starvation) that can approach the circadian range in supplemented cultures (Lakin-Thomas and Brody, 2000; Lakin-Thomas, 1998). To determine if the long-period rhythms observed without supplementation are driven by the same oscillator as the circadian develpmental rhythms observed in supplemented cultures, and to observe both oscillators simultaneously to determine if they are distinct, a codon-optimized luciferase was used to monitor the FRQ/WCC oscillator under conditions where the CDO controlled the conidiation rhythm (Shi et al., 2007) (see Fig. 10 for a description of the luciferase assay). Under conditions where the long-period, uncompensated, CDO-driven developmental rhythm was expressed for weeks in growth tubes, the luciferase rhythm in the same cultures continued in a typical compensated manner with a circadian period length dependent on the alleleic state of *frq*, and data analysis revealed no interaction between the uncompensated CDO and the circadian FRQ/WCC oscillator. Thus, it appears that the CDO can, under appropriate conditions, assume control of growth and development, thereby masking output from the circadian system. This approach will certainly be useful for dissecting the possible roles of other oscillatory systems within the circadian system.

OUTPUT FROM THE CIRCADIAN OSCILLATOR: THE HANDS OF THE CLOCK

The overt (observable) daily rhythms in organisms were what originally attracted the attention of biologists to the field of chronobiology. In *Neurospora* and other fungi, rhythms in several physiological properties have been described (Bell-Pedersen et al., 1996a; Lakin-Thomas et al., 1990), but it was the easily observable daily rhythm in the development of conidiospores that first led to the investigation of the *Neurospora* clock (Pittendrigh et al., 1959). The study of mutations that altered the pattern of rhythmic development was critical for the initial identification of FRQ/WCC oscillator components in *Neurospora* (Feldman et al., 1979), and the identification of genes that are regulated by the circadian clock has supported the existence of FRQ-independent oscillators in the *Neurospora* cell (Correa et al., 2003). Thus, the study of circadian output pathways not only has yielded information on what cellular functions are regulated by the clock and how this regulation occurs but also has been instrumental in the discovery of oscillator components.

Identification and Characterization of ccgs

To begin to characterize circadian output pathways at the molecular level in *Neurospora*, genes that are rhythmically expressed and thus likely under control of the clock were isolated. These studies were the first involving any organism to specifically target genes that have rhythms in mRNA

abundance, and the term clock-controlled gene (ccg) was used to describe them (Loros et al., 1989).

Standardized criteria have been used to define a rhythmically expressed gene as a ccg based on (i) demonstration of rhythmic expression or activity under constant conditions, (ii) demonstration that the period of the rhythm matched the genotype of the strain, and (iii) demonstration that inactivation of the gene had no effect on the function of the oscillator itself (Loros and Dunlap, 2001; Loros et al., 1989). The last criterion would distinguish an oscillator component that displays a rhythm from an output pathway component. However, this definition has become less useful as we learn more about the circadian clock as a system. For instance, examples of ccgs that feed back onto the oscillator or the input pathway have now been uncovered, which when inactivated affect the operation of the oscillator (Heintzen et al., 2001; Gwinner et al., 1997; Cassone et al., 1993; Heintzen et al., 1997; Pregueiro et al., 2006; Herzog and Block, 1999). Also, data suggest that more than one oscillator can regulate ccgs, so that a mutation in a component of one oscillator might not necessarily affect the period of a ccg regulated by a different circadian oscillator (de Paula et al., 2006).

In initial screens for ccgs in *Neurospora*, 12 rhythmic genes that peak in the late night to early morning were identified (Bell-Pedersen et al., 1996a; Loros et al., 1989; Zhu et al., 2001). In the long (29-h)-period frq^7 mutant strain, the period of the ccg mRNA rhythms was about 29 h (see Fig. 9 for an example). In all cases examined, the FRQ/WCC oscillator functioned normally in strains containing inactivated copies of the ccgs, demonstrating that they are part of, or at the end of, an output pathway and are not involved in oscillator function (Bell-Pedersen et al., 1992; Shinohara et al., 1998, 2002). These initial screens were followed some years later by the use of microarrays to globally screen for ccgs. To date, more than 150 ccgs have been identified in *Neurospora* by using microarrays that represented about one-seventh of the genome (Nowrousian et al., 2003; Correa et al., 2003). These studies have shown that the rhythmic genes function in a wide range of biological processes (Fig. 11). While the dominant peak in rhythmic expression for most of the ccgs anticipates dawn, the *Neurospora* clock regulates ccgs at all possible phases of the day (Correa et al., 2003).

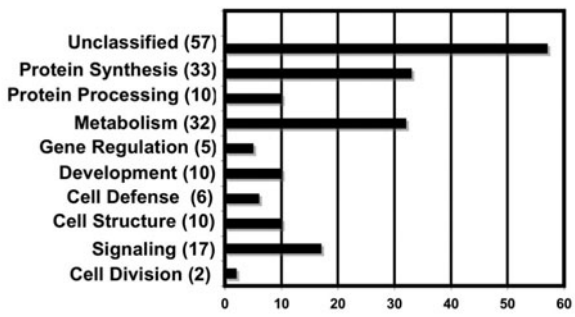

FIGURE 11 Functional classification of *Neurospora* ccgs. All of the known or predicted ccgs (182 ccgs) (Correa et al., 2003; Vitalini et al., 2006; Nowrousian et al., 2003) were classified according to their known or predicted functions from the Broad Institute *Neurospora* Sequencing Project (http://www.genome.wi.mit.edu/annotation/fungi/neurospora/). The number of genes in each category is in parentheses.

The Functions of the ccgs Are Providing Insights into the Role of the Clock in the Fungal Life Cycle

The predicted functions of the proteins encoded by the identified *Neurospora* ccgs have yielded insights into processes that are clock regulated, including roles in metabolism and stress responses. Genes directly involved in the expression of development have been identified, such as *ccg-2*, which encodes a hydrophobin protein found as an outer coat on asexual spores (Bell-Pedersen et al., 1992). Additional functions beyond development have also been identified as clock regulated (Vitalini et al., 2006; Correa et al., 2003). For example, several genes encoding enzymes involved in carbon and nitrogen metabolism show circadian rhythms in mRNA accumulation, with peaks occurring in the late night to early morning. In addition, the genes encoding glycogen phosphorylase, mannitol-1-phosphate dehydrogenase, and a low-affinity glucose transporter peak in the early night, suggesting that flux into the glycolytic pathway may crest at this time of day to prepare for increased energy requirements related to the consequent development of conidiospores. Several ccgs are involved in stress responses, including genes encoding antioxidant enzymes that prevent damage due to ROS are also under clock control, such as glutamate dehydrogenase, glutamine synthetase, oxidoreductase, and catalase. Genes encoding these enzymes peak in expression during the daytime, suggesting that the clock increases antioxidative defense mechanisms to cope with increases in free radicals that can result from light exposure. Other links to environmental stress include clock control of *ccg-1* transcription, a gene known to be induced by heat shock and hyperosmotic stress. *ccg-9* encodes trehalose synthase, which catalyzes the synthesis of the disaccharide trehalose; trehalose plays an important role in protecting cells from environmental stresses (Shinohara et al., 2002). Consistent with a role for *ccg-9* in stress responses, the transcript is induced by heat shock, glucose starvation, and osmotic stress. A role in development for *ccg-9* is also suggested by the finding that inactivation of *ccg-9* results in altered conidiophore morphology and abolishes the normal circadian rhythm of conidial development. *ccg-9*-null strains have normal FRQ cycling, phosphorylation, and light induction, indicating that loss of the conidiation rhythm may be a defect in circadian output and is not due to changes in either the FRQ/WCC oscillator or light input into the clock (Shinohara et al., 2002). *ccg-12* is identical to the *Neurospora cmt* gene encoding copper metallothionein involved in copper detoxification (Bell-Pedersen et al., 1996a). The *con* genes are preferentially expressed during conidiation but are not essential for development (Berlin and Yanofsky, 1985). Expression of *con-10* is under circadian clock control, and *con-10* encodes a small stress response protein of unknown function that is conserved in fungi. The *con-10* promoter has been studied in detail, and elements involved in its regulation by nitrogen or carbon starvation, heat shock, and light have been identified (Lee and Ebbole, 1998). The circadian regulation of *ccg-1*, *ccg-9*, *cmt*, and *con-10* supports a role for the clock in controlling stress responses in the organism. These genes, with the exception of *cmt*, are also induced during development. Clock control of these genes may have evolved as a way to anticipate stresses (e.g., increased mutagenic potential) and opportunities (e.g., increased temperature) in the 24-h LD cycle.

In support for a role of the clock in providing a mechanism for anticipating stress responses, it was recently shown that the *Neurospora* osmosensing mitogen-activated protein kinase (MAPK) pathway (the OS pathway), essential for osmotic stress responses, is a circadian output pathway that

regulates daily rhythms in expression of at least some of the downstream target genes of the pathway, including *ccg-1* and *ccg-9* (Vitalini et al., 2007). Temporal regulation of the OS pathway highlights the adaptive significance of the clock, as the ability of organisms to appropriately respond to changes in osmolarity is fundamental to survival. Consistent with this idea, global gene profiling in *Arabidopsis thaliana* revealed that approximately 68% of the ccgs overlap with genes that are differently regulated in response to osmotic and cold stresses (Kreps et al., 2002).

Signaling Time-of-Day Information from the Oscillator to the ccgs

A mechanism by which the output pathways are rhythmically controlled is at least in part through transcription factors that are themselves components of the oscillator (Fig. 12). These direct outputs would in turn regulate downstream ccgs. In *Neurospora*, the WCC transcription factor not only functions to turn on expression of the negative element FRQ but also signals time-of-day information directly from the oscillator to one or more output pathways to control the rhythmicity of downstream ccgs. For example, a C-Box has been identified in the promoters of several ccgs, including *vvd* and *al-3* (Carattoli et al., 1994; Correa et al., 2003; Bell-Pedersen et al., 2005), and the WCC was shown to bind directly to the promoters of *vvd* and *al-3* (He et al., 2005b). Not surprisingly, most of the C-Box-containing ccgs peak in the late night to early day, when the WCC is most abundant in the nucleus, supporting the idea that these genes are direct targets of the WCC. Four of the ccgs that are potential direct targets of the WCC are known or putative transcription or signaling factors that would be expected to control a subset of downstream ccgs. One target of these transcription factors may be an 8-nucleotide element (TCTTGGCA) occurring in about one-half of the *Neurospora* late-night-specific genes (Correa et al., 2003). This element is very similar to the core of a 45-bp fragment in the

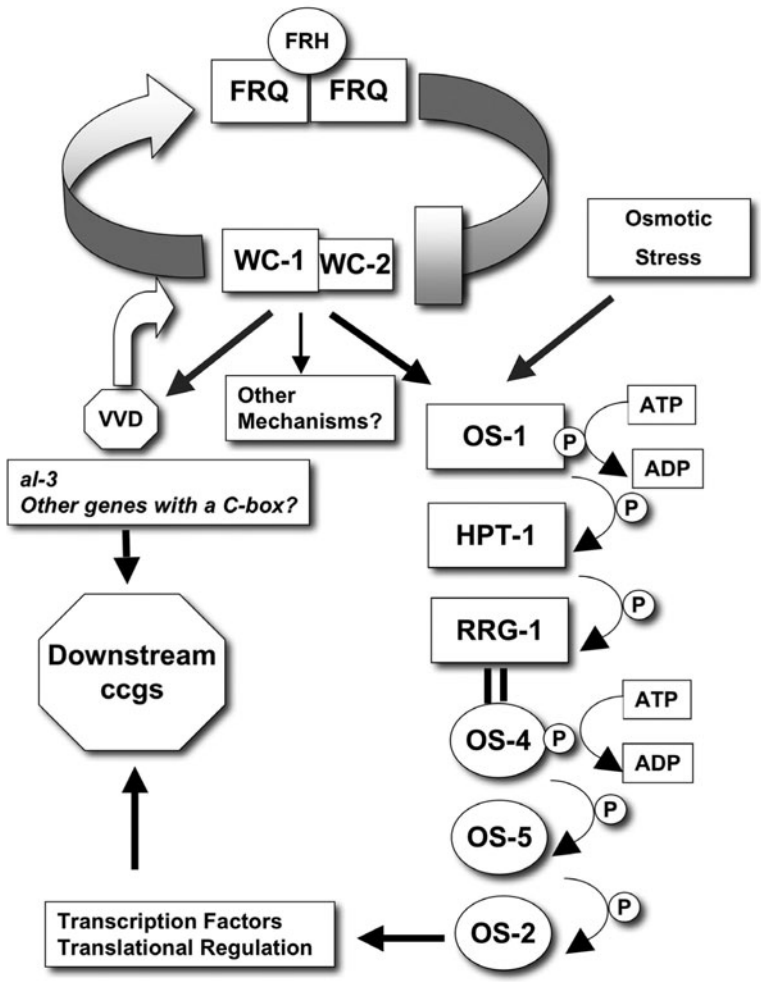

FIGURE 12 Model for the regulation of circadian output pathways in *Neurospora*. The WCC binds to the promoters of genes that lie within circadian output pathways, including *vvd*, *al-3*, and possibly other genes, including transcription factors that would control rhythmic expression of downstream target genes (Vitalini et al., 2006; He and Liu, 2005b). In addition, the FRQ/WCC oscillator signals to the osmotic-stress-sensing phosphorelay (the OS pathway) to control rhythms in OS-2 MAPK phosphorylation. Rhythmic phospho-OS would control rhythmic activity of target effector molecules, including transcription factors and factors involved in translation, which would, in turn, regulate downstream ccgs (de Paula et al., 2008). Together, these and other possible mechanisms control circadian output pathways that regulate rhythms in ccg expression responsible for overt rhythmicity in the organism.

ccg-2 promoter that is located near the start of transcription and contains a positive activating clock element (ACE) (Bell-Pedersen et al., 1996a). The ACE was shown to be both necessary and sufficient for rhythmicity, and factors are present in nuclear extracts that interact specifically with the ACE. The amount of binding and the mobility of the complexes change over the course of the day. Lastly, many ccgs lack both the ACE and the C-box, consistent with the existence of other clock control regulatory elements that may be direct targets of FRQ, regulated by other undescribed oscillator components, or regulated by the direct targets of the WCC.

Further progress in understanding how FRQ/WCC oscillator components signal time information through the output pathways to regulate rhythmic gene expression at different phases of the day came from the discovery of clock regulation of the OS MAPK signaling pathway (Vitalini et al., 2007). A model for clock control of this pathway is depicted in Fig. 12. In *Neurospora*, acute osmotic stress at any time of the day signals through a phosphorelay involving a sensor histidine kinase, OS-1, a histidine phosphotransferase, HPT-1, and a response regulator, RRG-1. This signal is then passed to the OS MAPK pathway, resulting in phosphorylation and activation of the MAPK OS-2. Phospho-OS-2 regulates downstream effector kinases, transcription factors, proteins involved in chromatin remodeling, and translation factors. Under constant growth conditions, the FRQ/WCC oscillator signals to the same pathway to regulate rhythmic phosphorylation of OS-2. Rhythmic phospho-OS-2 in turn controls rhythms in the effector molecules, which then regulate rhythmic expression of downstream target genes of the pathway, such as *ccg-1*, and *ccg-9* (Yamashita et al., 2008). Clock regulation of MAPK signaling pathways provides a mechanism to coordinately control major groups of genes such that they peak at the appropriate times of day to provide a growth and survival advantage to the organism, likely by anticipating stresses (de Paula et al., 2007).

Do Output Pathways Affect the Oscillator?

In several systems, output pathways feed back on the central oscillator or on the input to the oscillator. The modulating action of the clock-controlled component *VVD* on light input pathways (see above) provides an early example of how output pathways can feed back to influence the circadian oscillator (Elvin et al., 2005; Heintzen et al., 2001). In addition to *vvd*, the clock-controlled gene *prd-4* was shown to influence input to the FRQ/WCC oscillator in *Neurospora* (Pregueiro et al., 2006; Gardner and Feldman, 1981). Recall that *prd-4* is an ortholog of mammalian *checkpoint kinase 2* (*chk-2*). The original semidominant *prd-4* mutation displayed a short period length (18 h) at 25°C and a defect in temperature compensation. However, a *prd-4* deletion mutant has a period length similar to that of the wild type. This suggests that the original mutant allele (S493L) is a gain-of-function mutation and *prd-4* is not essential for clock function. *prd-4* mRNA accumulates rhythmically, peaking during the day. This regulation would allow cells to respond maximally to DNA damage caused by ionizing radiation during the day, and following PRD-4 phosphorylation in response to this damage would result in the arrest of the cell cycle. PRD-4 feeds back to the clock through an input pathway that signals DNA damage information to the FRQ/WCC oscillator via FRQ phosphorylation. The short-period phenotype of the *prd-4* mutant allele S493L is likely the result of premature phosphorylation of FRQ. DNA-damaging agents reset the clock, and this resetting requires PRD-4. This output-to-input loop involving *prd-4* is predicted to provide a time-of-day-specific "gate" during which DNA-damaging agents can affect the phase of the FRQ/WCC oscillator, which would in turn affect *prd-4* expression. In *Neurospora*, mutations that abolish conidiation at early stages do not abolish rhythmic aerial hypha formation, demonstrating that components of the conidiation output pathway do not feed back to the FRQ/WCC oscillator (Correa and Bell-Pedersen, 2002).

CIRCADIAN RHYTHMS IN OTHER FILAMENTOUS FUNGI

Circadian rhythms have been observed in several different fungal species but are primarily limited to field documentation of rhythms in spore development and liberation (Bell-Pedersen et al., 1996b). More recently, developmental and molecular rhythms in *Aspergillus* have been reported (Greene et al., 2003). In *Aspergillus flavus*, the clock was shown to control daily rhythms in the development of sclerotia, which are large survival structures produced by many fungi. This developmental rhythm exhibits all of the principal clock properties; the rhythm is maintained in constant environmental conditions with a period of about 30 h at 30°C, it can be entrained by environmental signals, and it is temperature compensated. Interestingly, this endogenous 30-h period is one of the longest natural circadian rhythms reported for any organism, and this likely contributes to some unique responses of the clock to environmental signals. In *Aspergillus nidulans*, no obvious rhythms in spore development were observed. However, a free-running and entrainable rhythm in the accumulation of *gpdA* mRNA (encoding glyceraldehyde 3-phosphate dehydrogenase) was shown, suggesting the presence of a circadian clock in this species.

Interestingly, *Aspergillus* lacks a detectable *frq* gene, suggesting that the *Aspergillus* clock differs from the *Neurospora* FRQ/WCC oscillator (Galagan et al., 2005; Machida et al., 2005; Nierman et al., 2005). However, homologs of *Neurospora wc-1* and *wc-2* are present in *A. nidulans*, *A. fumigatus*, and *A. flavus*. Because *Aspergillus* displays blue-light responses (Yager et al., 1998), it is possible that the primary force for maintaining the *wc* genes in the Ascomycetes was to allow blue-light sensing. It is also possible that the *Aspergillus* WC proteins function in FLO in a manner similar to that of the WC-FLO in *Neurospora*. The absence of FRQ, along with the unusual properties of the circadian clock in *A. flavus*, might suggest that the *Aspergillus* clock and *Neurospora* WC-FLO are related and that the WC-FLO is ancestral to the FLO. Ultimately, comparisons between the *Neurospora* and *Aspergillus* oscillators will allow investigation of whether circadian clocks have diverse evolutionary origins or whether molecular adornments have been added to a common ancestral mechanism.

SUMMARY

Solving the mechanisms of the circadian clock has become an important goal, mainly because of their ubiquity, their adaptive value, and their significance for health and disease in many organisms. The past several years have seen significant advances in our understanding of the mechanisms of circadian rhythmicity, with the molecular genetic analysis of clocks in *Neurospora* continuing to provide major insights into the molecular bases of circadian rhythmicity. The completed genome sequence, coupled with the use of new

technologies to identify additional components of the circadian system, is leading to a more detailed description of the processes that make up the circadian oscillator system, including novel oscillators and the genes that are regulated by the clock. In the future, these technologies will allow investigators to fully examine the effects of mutations in oscillator components and critical output regulators of *ccg*s in order to develop a detailed map of a circadian system and its interactions with other cellular pathways. While it is clear that different organisms use their clocks to regulate different biological processes, several fundamental aspects of rhythmicity are conserved, and an understanding of the circadian system in *Neurospora* will continue to provide important insights into the workings of the circadian clock for many years to come.

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NUTRIENT, pH, AND STRESS SENSING

VII

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Cellular and Molecular Biology of Filamentous Fungi
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How Fungi Sense Sugars, Alcohols, and Amino Acids

CHAOYANG XUE,* DANIEL J. EBBOLE, AND JOSEPH HEITMAN

SENSING SUGAR

Sensing Glucose

GPCRs and Glucose Sensing

Microbes adapt to the environment to obtain nutrients, including carbon and nitrogen sources, through sophisticated sensory mechanisms, and glucose is a major carbon and energy source for most cells. G-protein-coupled receptors (GPCRs) are an important receptor gene family and play important roles in sensing sugars in eukaryotic organisms, including fungi.

The first GPCR for a ligand other than mating pheromones identified in fungi was the glucose sensor Gpr1 from the baker's yeast *Saccharomyces cerevisiae*, which was isolated by screening a yeast two-hybrid library with the coupled Gα subunit Gpa2 (Xue et al., 1998). Gpr1 encodes a large protein containing over 800 amino acids with a long third cytoplasmic loop and a long C-terminal tail. Gpr1 and its homologs in other fungi share limited sequence homology with the other six GPCR classes that have been defined based on sequence homology and similarity in biological functions (Attwood and Findlay, 1994; Kolakowski, 1994), and they group as a novel evolutionarily distinct GPCR class. Gpr1 serves as a receptor to sense glucose and sucrose and activates the G protein α subunit Gpa2, which in turn activates adenylyl cyclase to convert ATP into cyclic AMP (cAMP) (Kraakman et al., 1999; Lemaire et al., 2004; Lorenz et al., 2000b; Rolland et al., 2002; Xue et al., 1998). cAMP then binds to the regulatory subunit of protein kinase A (PKA), Bcy1, and thereby releases the catalytic subunits of PKA (Tpk1, 2, and 3) to phosphorylate downstream target proteins and influence pseudohyphal differentiation and cellular metabolism and physiology (Fig. 1). Interestingly, the affinity of Gpr1 for sucrose is much higher than for glucose. Approximately 20 to 30 mM glucose is required to half-maximally activate (50% effective concentration) Gpr1-dependent cAMP signaling in vivo, while the 50% effective concentration for sucrose is much lower (0.5 mM) (Lemaire et al., 2004; Versele et al., 2001). Testing of other sugars with similar structures (such as galactose, mannose, and fructose) suggests that mannose acts as an antagonist for both sucrose and glucose, while others had no effect (Lemaire et al., 2004). In addition to being directly sensed by Gpr1 as an extracellular signaling molecule, glucose can also be transported into the cell via hexose transporters and converted to glucose-6-phosphate through phosphorylation to undergo glycolysis. Glucose-6-phosphate can also trigger a transient cAMP elevation without the involvement of Gpr1, which suggests that glucose-6-phosphate may also function as a signaling molecule for cAMP pathway activation (Rolland et al., 2000, 2001). Activation of adenylyl cyclase and downstream signaling may also be influenced by other G protein systems, such as the Ras proteins (Colombo et al., 2004).

The Gpr1 protein sequence is conserved in other ascomycetes, and its homologs have been studied in several other yeasts, including the pathogenic yeast *Candida albicans* and the model fission yeast *Schizosaccharomyces pombe*. The *C. albicans* Gpr1 receptor is important for filamentous growth and to activate the cAMP-regulated PKA signaling pathway via direct interaction with Gpa2, similar to what is observed with *S. cerevisiae* (Maidan et al., 2005a; Miwa et al., 2004). However, the role of *Candida* Gpr1 in glucose sensing remains unclear. Some phenotypic analysis and biochemical studies suggested that Gpr1 was involved in the glucose-sensing machinery that regulates morphogenesis and hypha formation on solid media but found it to be dispensable for hypha formation in liquid medium or during host infection (Miwa et al., 2004). Independent studies showed that Gpr1 and Gpa2 have no effect on glucose-induced cAMP signaling and may not be involved in glucose sensing (Maidan et al., 2005a). Instead, deletion mutants of Cdc25 or Ras2 were found to abolish glucose-induced cAMP signaling, suggesting that the Cdc25-Ras2 branch is instead responsible for glucose sensing in *C. albicans*. More recent evidence suggests that Gpr1 senses amino acids, such as methionine. Methionine triggers Gpr1 internalization and

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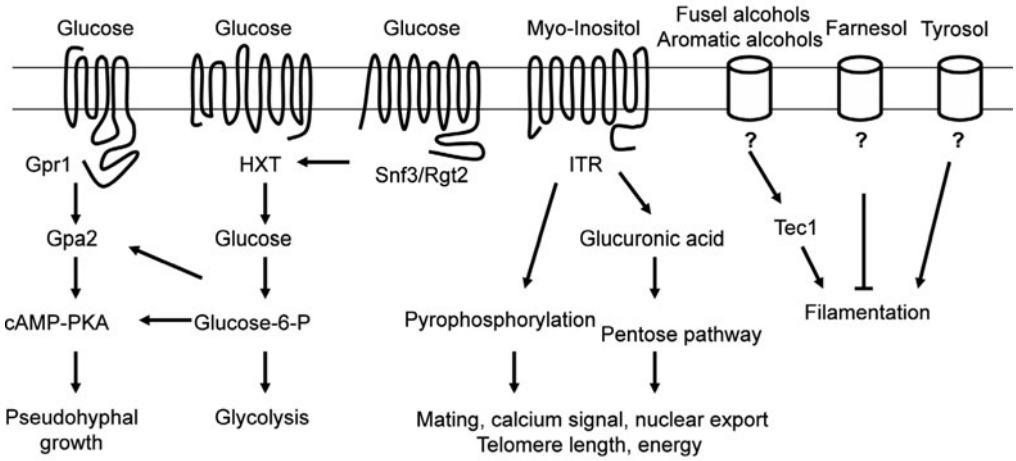

FIGURE 1 Carbon sensors in yeast. *S. cerevisiae* utilizes multiple sensory systems to sense and import carbon sources from the environment. A Gpr1 GPCR system senses extracellular sugars and activates the Gpa2-cAMP-PKA signaling pathway to control pseudohyphal differentiation. Glucose can also be sensed by a transporter-like receptor system that involves Snf3 and Rgt2, which regulate the expression of the major hexose transporter gene family (HXT). The HXT proteins import glucose to undergo glycolysis, and its intermediate, glucose-6-phosphate, also triggers the activation of cAMP-PKA signaling. Yeasts may also sense *myo*-inositol through the *myo*-inositol transporter gene family and utilize inositol as a precursor for many metabolic and catabolic processes that play critical roles in signaling regulation and cell developmental processes. A variety of alcohol-related products (such as fusel alcohols, aromatic alcohols, and farnesol-related molecules) that are produced by yeasts themselves provide an autoregulatory machinery to regulate yeast cell population and filamentation processes.

promotes hypha formation on solid media in a Gpr1-dependent fashion (Maidan et al., 2005a). However, the effect of methionine on hypha formation requires the presence of a carbon source such as glucose in the medium, and methionine has not been found to induce cAMP production in *C. albicans*. Therefore, it remains unclear whether Gpr1 also senses sugars, as in *S. cerevisiae*, only senses methionine, or senses both methionine and glucose (Maidan et al., 2005b).

Git3 is a Gpr1 homolog in the fission yeast *S. pombe*, and it functions as a glucose sensor to activate a heterotrimeric G protein composed of the Gα Gpa2, the Gβ Git5, and the Gγ Git11 subunits. Interestingly, in addition to interacting with Gα Gpa2 and regulating cAMP signaling, as in *S. cerevisiae*, when Git3 was fused to the Gpa1 Gα subunit it was able to transiently activate the pheromone pathway in response to glucose induction (Hoffman, 2005). Thus, Git3 appears to be an authentic glucose sensor and plays important roles in nutrient sensing that are coordinately involved in regulating mating with the pheromone response pathway (Hoffman, 2005; Welton and Hoffman, 2000).

Gpr1 homologs have also been reported in several filamentous fungi, such as *Neurospora crassa* (Li and Borkovich, 2006), *Aspergillus nidulans* (Han et al., 2004), and *Magnaporthe grisea* (Kulkarni et al., 2005). In *N. crassa*, the Gpr1 homolog GPR-4 was found to function upstream of the Gα subunit GNA-1 based on genetic epistasis analysis. A physical interaction between the GPR-4 C-terminal domain and GNA-1 was also detected in the yeast two-hybrid assay, which suggests that GPR-4 may be a receptor for GNA-1 involved in cAMP signaling, since GNA-1 is required for the activation of this pathway. *gpr-4* mutants exhibit defects in growth/dry mass, and this defect is partially rescued by exogenous cAMP. The observations that glucose induces cAMP production and *gpr-4* mutations block this stimulation further indicate that GPR-4 plays a role in glucose sensing and cAMP signaling activation. However, steady-state cAMP levels are similar in wild-type and *gpr-4* mutant strains, suggesting that glucose may only promote transient or localized cAMP induction via GPR-4. These results provide evidence that GPR-4 may function as a glucose sensor to activate the glucose-regulated cAMP signaling pathway via coupling to GNA-1 in *N. crassa* (Li and Borkovich, 2006). It remains to be understood how GPR-4 senses glucose and how it regulates cAMP signaling at a biochemical level. In *A. nidulans*, three Gpr1 homologs (GprC, GprD, and GprE) have been identified, but it is still unknown whether these GPCRs play a role in glucose sensing. Functional studies revealed GprD as a negative regulator of sexual development that also controls vegetative growth (Han et al., 2004). A large group of GPCR-like 7-TM proteins have been identified in *M. grisea* based on bioinformatic analysis, including the Gpr1 homolog MGG_08803, but its function remains to be analyzed (Kulkarni et al., 2005).

No direct Gpr1 glucose receptor homolog has been identified in basidiomycetes. How these fungi sense sugars remains to be elucidated. Glucose is a preferred carbon source for *Cryptococcus neoformans* and activates the Gpa1-cAMP signal pathway, which is conserved with the cAMP signaling cascade in *S. cerevisiae*. A group of 7-TM proteins has been identified in *C. neoformans*, and the Gpr4 receptor shares structural similarity rather than sequence identity with Gpr1 from *S. cerevisiae* and *C. albicans*. Similar to Gpr1, Gpr4 encodes a large protein containing over 800 amino acids with a long third cytoplasmic loop and C-terminal tail; however, Gpr4 is not important for glucose sensing but rather is involved in amino acid sensing, similar to Gpr1 in *C. albicans* (Xue et al., 2006).

Sugar Transporter Homologs as Sugar Sensors

In *S. cerevisiae*, sugar sensing involves the glucose transporter homologs Snf3 and Rgt2. These transporter homologs serve

as receptors for precisely measuring the extracellular concentration of glucose (Fig. 1). Both proteins have C-terminal cytoplasmic tails that interact with casein kinase I (Yck1 and Yck2) and with two paralogous proteins (Mth1 and Std1). In the absence of glucose, Mth1 and Std1 serve as corepressors with the DNA binding protein Rgt1 to repress hexose transporter gene expression (Moriya and Johnston, 2004). Although Snf3 and Rgt2 do not themselves transport glucose (Ozcan et al., 1998), it is thought that binding of glucose leads to a conformational change in the transporter that activates casein kinase I. This leads to phosphorylation of Mth1 and Std1, targeting them for degradation. Depletion of the corepressors relieves repression of hexose transporter transcription. Hexose transporter genes are the primary regulatory targets of this glucose-sensing mechanism in yeast, and this regulation optimizes the levels of high- and low-affinity hexose transporter expression (Fig. 1).

Interestingly, the cytoplasmic tails of Rgt2 and Snf3 can be fused to other sugar transporters, and overexpression of these chimeric proteins is able to derepress expression of hexose transporter genes (Ozcan et al., 1998). Furthermore, overexpression of a membrane targeted form of the cytoplasmic tail alone leads to constitutive activation of the sensing pathway (Dlugai et al., 2001). Thus, it is likely that a high concentration of the Snf3 or Rgt2 cytoplasmic tail at the membrane is sufficient to cause signaling by bringing Mth1 and Std1 to Yck1 in the membrane. However, overexpression of an Rgt2 allele lacking the tail also activates hexose transporter (Hxt1) expression (Moriya and Johnston, 2004). This suggests that Rgt2 may stimulate Yck1 through an interaction independent of Yck1 binding to the cytoplasmic tail of Rgt2.

Other yeasts appear to have sugar transporter homologs that act as sensors in a way similar to that of Snf3 and Rgt2, notably, *Kluyveromyces*, *Pichia*, and *Candida* (Gancedo, 2008). Interestingly, recent studies revealed that *C. albicans* lacks the Gal sensing system that is used for galactose sensing in *S. cerevisiae*, and instead this pathogenic yeast utilizes a hexose transporter homologue, Hgt4, to sense galactose (Brown et al., 2009). This may be relevant to how and where *C. albicans* acquires carbon sources as part of the normal human microbiota growing in competition with numerous bacterial species.

In the Euascomycetes, *N. crassa* has been shown to possess a sugar transporter homolog with features of a sugar sensor. A gene, *rco-3*, was discovered in several independent mutant screens for sorbose resistance (*sor-4*) and deoxyglucose resistance (*dgr-3*) (Allen et al., 1989) and for mutants derepressed for sporulation (*rco-3*) (Madi et al., 1994).

The *rco-3* gene is predicted to encode a 594-amino-acid protein with 37% identity to Snf3 in the region homologous to sugar transporters. RCO-3 contains a short C-terminal extension beyond the transporter homologous region that lacks sequence similarity to the cytoplasmic tails of Snf3 and Rgt2. The RCO-3 tail does contain a glutamine-rich region (19 of 26 residues) followed by a serine- and threonine-rich stretch (10 of 15 residues). There are clear orthologs of *rco-3* among the Euascomycetes, although the sequences of the C-terminal tails are not conserved and many do not have clear C-terminal tails. The *rco-3* transcript has been difficult to detect using Northern blot experiments. In addition, the *rco-3* cDNA initiates 402 nucleotides upstream of the RCO-3 start codon and contains a 22-amino-acid open reading frame initiating 103 nucleotides upstream of the RCO-3 start codon. The level of mRNA abundance may not reflect the translation rate, since upstream open reading frames are known to reduce translation of downstream coding regions. Taken together, these findings suggest that the RCO-3 protein is not produced in abundance in *Neurospora*.

The *rco-3* mutant has a slightly reduced growth rate on agar medium with 2% glucose as the carbon source (4.1 mm/h for *rco-3* versus 4.8 mm/h for the wild type). However, the linear growth rate on 2% fructose (4.4 mm/h), xylose (4.2 mm/h), or glycerol (2.9 mm/h) is indistinguishable from that of the wild type (Madi et al., 1997). This suggests that glucose transport activity is limiting for growth of the *rco-3* mutant. Despite the wild-type level of growth on fructose, xylose, and glycerol, the *rco-3* mutant is derepressed for asexual development.

Neurospora has a glucose-repressible high-affinity glucose transport system (K_m = 0.01 to 0.07 mM) and a low-affinity transport system ($K_m \sim$ 20 mM) (Allen et al., 1989). In the yeast *S. cerevisiae*, the high-affinity transport system has an apparent K_m of 1 mM (Coons et al., 1995). The *rco-3* mutant is partially derepressed in high-affinity glucose transport activity in the presence of glucose and is not fully derepressed by glucose limitation. In addition, *rco-3* mutants have reduced low-affinity glucose transport activity (Allen et al., 1989; Madi et al., 1997). Thus, the deoxyglucose and sorbose resistance phenotypes and the low growth rate on glucose medium may result from low hexose transport activity.

Does RCO-3 regulate genes in addition to sugar transporters? Quinic acid utilization genes are repressed by glucose, and the *qa-2* gene (dehydroquinase) is not glucose repressible in *rco-3* mutants. Phosphoenolpyruvate carboxykinase (PEPCK) is the rate-controlling enzyme in gluconeogenesis, and even low levels of extracellular glucose repress its expression. PEPCK is derepressed in the *rco-3* mutant. A glucose-regulated gene (*grg-1*/*ccg-1*) and *hgt-1*, a high-affinity glucose transporter gene (Xin et al., 2004), are similarly derepressed in *rco-3* mutants.

The *rco-3* mutant is defective in regulation of both high- and low-affinity glucose transport and glucose repression of a number of other genes, suggesting that RCO-3 serves as a sensor of glucose. In addition, *rco-3* mutants are not inhibited for conidiation by a number of different carbon sources. RCO-3 may be a sensor of glucose that regulates genes controlling development directly, rather than an indirect regulator of development through its effects on sugar transporter expression and sugar uptake. Sugar sensors in the Euascomycetes may play a larger role in behavior than has been observed in *S. cerevisiae*. Mutants with similar phenotypes (Allen et al., 1989) may define genes for other sugar sensors or components of the *rco-3* signaling pathway.

Sensing Other Carbon Sources

Inositol Sensing and Metabolism

Inositol can serve as a carbon source for yeasts and also plays an important role in development of some filamentous fungi, such as *N. crassa*. *myo*-Inositol metabolic and catabolic pathways have been extensively studied in *S. cerevisiae*, and many downstream intermediate metabolites have been identified that are involved in a plethora of cell signaling and cell developmental processes, including calcium signaling, osmotic stress, nuclear export, telomere length, and PKA signaling (Seeds and York, 2007).

The *myo*-inositol transporter gene family is part of the sugar transporter superfamily and may also play important roles in *myo*-inositol sensing in fungi (Fig. 1). High sequence similarity within this gene family suggests that these genes likely evolved from a common ancestor. There are two *myo*-inositol transporters (Itrs) in *S. cerevisiae*, which were first

isolated by complementation of a yeast mutant defective in *myo*-inositol uptake. Itr1 is the major transporter and is transcriptionally and posttranslationally repressed by inositol and choline. Itr2 is a minor transporter that is constitutively expressed (Nikawa et al., 1991, 1993). Depletion of *myo*-inositol from the growth medium stimulates Itr1 expression, while addition of inositol to the medium triggers repression of Itr1 expression and inhibits uptake activity. Inactivation of the Itr1 permease triggers endocytic internalization and vacuolar degradation of the protein, but endocytosis is not required for normal inositol uptake (Lai et al., 1995).

Inositol also plays an important role in *C. albicans*. Inositol functions as an essential precursor for phospholipomannan, a glycophosphatidylinositol-anchored glycolipid on the cell surface of *Candida* that binds to human macrophages and is important for pathogenicity. In addition, inositol plays an essential role in the phosphatidylinositol signal transduction pathway, which controls cell cycle events, similar to *S. cerevisiae*. Two proton-coupled *myo*-inositol transporters have been identified. *C. albicans* Itr1 exhibits high substrate specificity for inositol and was not significantly affected by other closely related hexose or pentose sugars as competitors. Interactions between the C-2, C-3, and C-4 hydroxyl groups of *myo*-inositol and the transporter are critical for substrate recognition and binding (Jin and Seyfang, 2003). A recent study showed that, similar to *S. cerevisiae*, *C. albicans* can generate inositol de novo through Ino1 and also import it from the environment through the *myo*-inositol transporter Itr1. *C. albicans* may utilize these two complementary mechanisms to obtain inositol during host infection (Chen et al., 2008; Reynolds, 2009).

The fission yeast *S. pombe* is a natural auxotroph for inositol due to the absence of inositol-1-phosphate synthase (Ino1) and cannot grow in the absence of inositol. A low concentration of inositol in the culture medium supports vegetative growth but not mating and sporulation, which require higher inositol concentrations (Niederberger et al., 1998). It was found that *myo*-inositol regulates the production of pheromone P and the response of cells to pheromones, but production of pheromone M is inositol independent. It is likely that inositol or one of its metabolites is involved in pheromone P secretion and pheromone signaling and thereby influences sexual reproduction (Voicu et al., 2002). *myo*-Inositol in *S. pombe* is also transported by the *myo*-inositol transporters Itr1 and Itr2, but whether either of these proteins also senses inositol is unknown.

C. neoformans is one of a few yeasts that can use inositol as a sole carbon source (Healy et al., 1977). *C. neoformans* is an opportunistic fungal pathogen capable of catabolizing and synthesizing inositol, and during infection this organism preferentially localizes to the inositol-rich environment of the central nervous system (Vincent and Klig, 1995). Inositol metabolism is important for growth and development of *C. neoformans* and may be involved in this organism's mechanism for survival, both in environmental niches such as plants and soil and in humans and other hosts (Franzot and Doering, 1999; Xue et al., 2007). One key enzyme for inositol catabolism is inositol oxygenase, which converts inositol into glucuronic acid in the presence of molecular oxygen. This enzyme has been purified from the related yeast *Cryptococcus lactativorus*, and biochemical characterization indicates that *myo*-inositol is the only accepted substrate for this enzyme (Kanter et al., 2003).

Our recent studies on the interactions between *Cryptococcus* and plants, a major environmental niche, reveal that *myo*-inositol, produced and secreted by plants, is sensed by the fungus and promotes fungal sexual reproduction, providing a potential explanation for how this organism completes its life cycle in nature (Xue et al., 2007). We have also discovered a defined medium, MS, which supports robust mating of both *C. neoformans* and *Cryptococcus gattii*. By omission and readdition of components, *myo*-inositol was shown to be the active component that stimulates mating. Notably, the standard V8 mating medium is known to contain ~17 mg/liter inositol, and this level is sufficient to support mating in MS medium. An expanded *myo*-inositol transporter (*ITR*) gene family has been identified as potential inositol sensors in *Cryptococcus*. This gene family contains at least seven members compared to only two in many fungi, such as *S. cerevisiae*, *S. pombe*, and the corn smut fungus *Ustilago maydis*. Gene mutagenesis studies reveal that the Itr1 transporter is important for mating on media containing inositol, and expression of several additional inositol transporters and *myo*-inositol oxygenases are upregulated by *myo*-inositol (our unpublished data). It is unclear at present whether any of these *myo*-inositol transporters function as a sensor, similar to the glucose-sensing glucose permease homologs Snf3 and Rgt2 in budding yeast.

The involvement of *myo*-inositol metabolism in regulating Ca^{2+} levels has been extensively studied in fungi. Two Ca^{2+} release pathways in *C. albicans* that require the function of vacuolar membrane vesicles have been revealed: one is induced by inositol 1,4,5-trisphosphate (IP_3) and the other by inside-positive voltage. The IP_3-gated pathway exhibits high affinity for IP_3 but is not activated by inositol 4,5-bisphosphate (IP_2) or inositol 1,3,4,5-tetrakisphosphate (IP_4) (Calvert and Sanders, 1995).

In filamentous fungi, hyphal extension requires a Ca^{2+} gradient that is highest at the hyphal tips. Based on studies of *N. crassa*, this tip-high Ca^{2+} gradient is generated and maintained internally by IP_3-induced Ca^{2+} release from tip-localized vesicles and subapical Ca^{2+} sequestration. There are two types of IP_3-activated Ca^{2+} channels in *Neurospora* membranes with different conductances: a low-conductance channel, localized to the endoplasmic reticulum and plasma membrane, and a high-conductance channel, localized on vacuolar membranes (Silverman-Gavrila and Lew, 2002, 2003). Similar mechanisms may also be involved in Ca^{2+} level regulation in other filamentous fungi, such as *M. grisea* and *Phyllosticta ampelicida*, a causal agent of grape black rot. These two fungi produce a special structure called an appressorium, and appressorium formation is regulated by cell calcium levels, which can be influenced by *myo*-inositol uptake (Lee and Lee, 1998; Shaw and Hoch, 2000).

Sensing Alcohol-Related Carbon Sources

S. cerevisiae has been found to sense fusel alcohols, the end products of amino acid catabolism, to regulate differentiation (Chen and Fink, 2006; Dickinson, 1996; Lorenz et al., 2000a) (Fig. 1). Diploid yeast cells typically differentiate into a filamentous growth form known as pseudohyphae under nitrogen starvation conditions. Several fusel alcohols, such as 1-butanol and isoamyl alcohol, stimulate filamentous growth of haploid cells. Filamentous growth involves binding of the transcription factor Ste12 to filamentation-specific genes in a Tec1-dependent but pheromone-responsive mitogen-activated protein kinase (MAPK)-independent mechanism, which is different from the nutrient-sensing machinery involving Gpr1-Gpa2-cAMP signaling (Zeitlinger et al., 2003). Fusel alcohols have also been found to inhibit yeast cell growth by inhibiting the initiation of eIF2B-dependent translational processes in a strain-specific manner (Ashe et al., 2001; Smirnova et al., 2005). Butanol also induces pseudohyphal morphology, even in liquid medium, in a mechanism that involves the Swe1-dependent morphogenesis checkpoint and differs from nitrogen-limitation-induced

pseudohypha formation (Martinez-Anaya et al., 2003). Ethanol was also found to stimulate hyperfilamentation in diploid cells in an MAPK-dependent manner (Lorenz et al., 2000a). Dickinson recently proposed that fusel-alcohol-induced filamentation is a response to impaired cell growth (Dickinson, 2008).

A recent study showed that aromatic alcohols (such as tryptophol and phenylethanol) secreted by yeast cells function as quorum-sensing molecules and stimulate filamentous growth through a Flo11-dependent mechanism (Chen and Fink, 2006). The production of these signaling alcohols is regulated by nitrogen level: high ammonia represses the expression of their biosynthetic pathway, whereas nitrogen-poor conditions (such as ammonium-limiting conditions) activate it. Interestingly, production of these aromatic alcohols is also controlled by cell density and autoregulated via a positive-feedback response involving the transcript factor Aro80. These interactions define a quorum-sensing circuit that allows *Saccharomyces* to respond to both cell density and nutritional conditions of the surrounding environment. The function of these autoregulatory molecules appears to be species specific, since they do not evoke the morphological switch in *C. albicans* (Chen and Fink, 2006).

Other alcohol-related quorum-sensing molecules, such as farnesol and tyrosol, have been extensively studied, especially in *Candida* species (Hogan, 2006; Nickerson et al., 2006; Zhang and Dong, 2004). Quorum-sensing molecules were first studied in bacteria and later were discovered in fungi. Farnesol accumulates when *C. albicans* yeast cells reach early stationary phase, triggering inhibition of both yeast growth and filamentation. Resuspending yeasts in fresh medium reverses this inhibition. Transcriptional profile analysis of yeast cells exposed to farnesol revealed that genes involved in hyphal formation, GTPase activation, mitosis, and DNA replication were downregulated, consistent with the cell growth inhibitory effects (Uppuluri et al., 2007). In contrast to inhibition of germ tube formation by farnesol, tyrosol is released into the medium continuously during growth and stimulates the formation of filamentous protrusions. The quorum-sensing process is therefore under complex positive and negative regulation by environmental conditions (Chen et al., 2004). Farnesol is also a virulence factor and inhibits production of both interleukin-12 p40 and p70 from gamma interferon/lipopolysaccharide-stimulated macrophages during fungal-host interactions (Navarathna et al., 2007).

The study of the effects of farnesol in the filamentous fungus *A. nidulans* revealed an interesting interaction between fungi (Semighini et al., 2006). External farnesol triggers apoptosis-like morphological features, which involves mitochondrial function and reactive oxygen species (ROS) production. In contrast to what occurs in *Candida*, there is no effect on *Aspergillus* hyphal morphogenesis. Moreover, the effects of farnesol appear to be mediated by the FadA heterotrimeric G protein complex. Because *A. nidulans* does not secrete detectable amounts of farnesol, most likely the apoptosis triggered by farnesol is a consequence of sensing farnesol produced by other fungi, such as *C. albicans*. In agreement with this notion, growth and development were restricted in a farnesol-dependent manner when *A. nidulans* was cocultivated with *C. albicans*. These results reveal that quorum-sensing molecules can be deployed by one species such as *C. albicans* to reduce growth of other competing microbes such as *A. nidulans* during their encounters in nature (Semighini et al., 2006).

So far, it is not clear how fungal cells sense any of these quorum-sensing molecules. One study has provided evidence that the two-component system signaling transduction pathway involving the Chk1 histidine kinase is important for sensing farnesol (Kruppa et al., 2004).

SENSING AMINO ACIDS

Amino acids are important nutrients for fungi and are detected by specialized sensor systems, which include the general amino acid permease Gap1, the Ssy1-Ptr3-Ssy5 (SPS) system, GPCRs, and the target of rapamycin (TOR) (Bahn et al., 2007). A new transporter sensor system that senses small peptides has also been recently identified (Fig. 2).

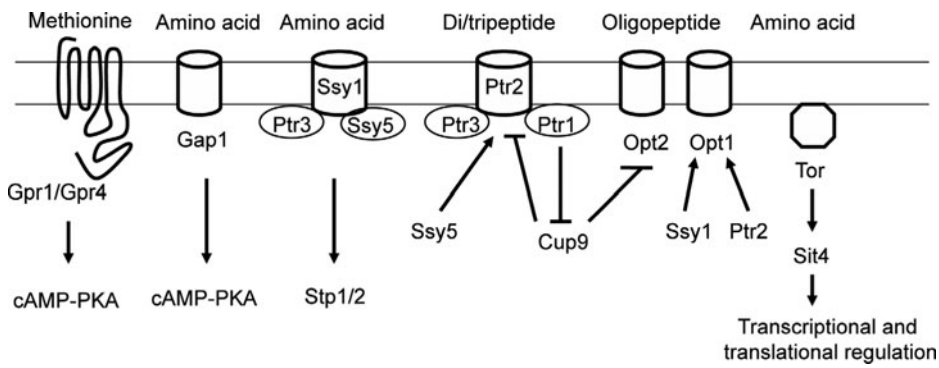

FIGURE 2 Amino acid sensory systems. Fungal cells require nitrogen sources, including amino acids, from the extracellular environment through a sophisticated sensory network, which involves the general amino acid permease Gap1 to uptake a broad range of amino acids, the SPS system to sense more specific amino acids groups, and two permease systems to respond to oligopeptides, including the PTR system, which imports di/tripeptides, and the OPT system, which transports oligopeptides. This amino acid permease network is highly coordinated through regulation between members and some common regulators, such as Cup9. In addition to the amino acid permeases, GPCR receptors that sense certain amino acids as signaling molecules have also been identified in the pathogenic yeasts *C. albicans* and *C. neoformans*. Also, the Tor protein complex is important for sensing nutrient signals, including amino acids, and regulating the stability of some amino acid permeases, as well as many transcriptional and translational processes.

General Amino Acid Permease Gap1

The general amino acid permease (Gap1) of *S. cerevisiae* is a low-affinity permease that imports a broad range of amino acids in cells grown under nitrogen-limiting conditions (Jauniaux and Grenson, 1990). This permease is also required for cells to respond to surrounding nitrogen sources via the PKA signaling pathway (Donaton et al., 2003). When ammonium is added to yeast cells growing with proline as the only nitrogen source, Gap1 is rapidly internalized and degraded via a pathway involving the Npi1/Rsp5 ubiquitin ligase (Springael and Andre, 1998). Gap1 biogenesis is coupled to sphingolipid biosynthesis, which creates a sphingolipid microenvironment essential for the normal conformation, function, and ubiquitination of Gap1 (Lauwers et al., 2007). The function of Gap1 has been linked to the PKA pathway (Donaton et al., 2003), and the Ras2/cAMP/PKA pathway may be involved in ubiquitin-dependent degradation of Gap1 (Garrett, 2008).

In *N. crassa*, three transport systems have been described based on the analysis of the kinetics of amino acid uptake and the patterns of competitive inhibition between amino acids. The Gap1 homolog in *N. crassa* is encoded by the *PMG* locus, which can transport all L-amino acids except proline (Margolis-Clark et al., 2001; Pall, 1969). Besides this system, two other transport systems have also been identified. One is encoded by the *MTR* gene and transports neutral and aromatic amino acids (Pall, 1969). The other is encoded by the *PMB* gene and transports basic amino acids, such as arginine and lysine (Pall, 1970).

The SPS System and Amino Acid Sensing

Another sophisticated amino acid sensor system has been identified in fungi, in addition to the general amino acid permease Gap1. In *S. cerevisiae*, the SPS sensor system plays an important role in amino acid sensing and transport (Didion et al., 1998; Forsberg and Ljungdahl, 2001; Klasson et al., 1999; Wu et al., 2006). In this system, Ssy1 is a membrane protein resembling an amino acid permease that functions instead as an amino acid sensor, similar to the role of Snf3 and Rgt2 in glucose sensing. Ptr3 and Ssy5 function downstream of Ssy1 and physically interact with Ssy1 to form a signaling complex (Forsberg and Ljungdahl, 2001). Binding of amino acids to Ssy1 activates the Ssy5 protease, which in turn proteolytically activates the latent transcription factors Stp1 and Stp2 to induce expression of genes encoding amino-acid-metabolizing enzymes and amino acid permeases (Andreasson et al., 2006). Ptr3 is a phosphoprotein and is hyperphosphorylated in response to extracellular amino acids in an Ssy1-dependent, Ssy5-independent manner. Recently, an SCF E3 ligase complex was identified to be important for Ptr3 function. Deletion mutations of a component of this E3 ligase complex, Grr1, block amino-acid-induced Ptr3 hyperphosphorylation (Liu et al., 2008). Studies on the SPS amino acid sensor system have mostly focused on *S. cerevisiae*, but Ssy1, Ptr3, and Ssy5 homologs in *C. albicans* have been reported to function in a pathway that senses amino acids and is important for virulence in the host (Brega et al., 2004; Martinez and Ljungdahl, 2005). It will be of interest to investigate the impact of this sensory system on the virulence of other plant and human fungal pathogens.

Transporters Sense Small Peptides

In *S. cerevisiae*, two transport systems are responsible for transporting small peptides (2 to 5 amino acids) into cells: PTR (acronym for peptide transport) for di-/tripeptides and OPT (acronym for oligopeptide transport) for oligopeptides of 4 to 5 amino acids in length. The PTR system consists of three proteins, Ptr1, Ptr2, and Ptr3. Ptr2 is a transporter that translocates both di- and tripeptides. Ptr1 contains ubiquitin-protein ligase (E3) activity and regulates the expression of Ptr2 by degrading the homeodomain protein Cup9, which represses Ptr2 by binding to its promoter (Byrd, et al., 1998). Ptr1 is not required for the expression of Ptr3, which also regulates Ptr2 expression and is also a member of the SPS amino acid sensing system. The function of Ptr2 is regulated not only by Ptr1 and Ptr3 but also by components of the SPS system, such as Ssy1 (Hauser et al., 2001).

The OPT system was first reported in *C. albicans* by the identification of Opt1 and found to be involved in the transport of tetra- and pentapeptides (Lubkowitz et al., 1998). To date, eight members of the *OPT* gene family have been described, and five of them encode proteins with oligopeptide-transporting activity, while the function for the remaining three is still unclear (Reuss and Moerschhäuser, 2006). It is unknown whether any of these proteins functions as a sensor instead of a transporter, similar to the glucose sensors Snf3 and Rgt2. Three members of the OPT system have been identified in *S. cerevisiae*. So far, only Opt1 has been studied in detail, and it is upregulated by naturally occurring amino acids under sulfur starvation conditions. Both Ptr3 and Ssy1 also regulate the expression of Opt1. The Ptr2 repressor Cup9 is also a repressor of Opt2, but not Opt1. These interconnected regulation patterns indicate that these amino acid sensors and transport systems are highly functionally coordinated, which allows cells to adapt utilization of amino acids or small peptides to various environmental conditions (Wiles et al., 2006).

A small peptide that controls a density-dependent growth phenotype reminiscent of quorum sensing was uncovered based on studies of the global repressor Tup1 in *C. neoformans* (Lee et al., 2005, 2007). At an inoculum size of $<10^3$ cells, the *tup1* mutant strain fails to form colonies on agar media, while at an inoculum of 10^5 to 10^6 cells per plate colonies are produced as an abundant lawn. This phenotype, expressed as an inability to grow at low cell densities, can be rescued by culture filtrate from a high-cell-density *tup1* mutant culture. The active compound in this culture filtrate was identified as an oligopeptide composed of 11 amino acids (NH_2-NFGAPGGAYPW-COOH). Compared to the recent identification of a variety of alcohols as quorum-sensing molecules, the characterization of this small peptide as an autoregulatory molecule suggests that diverse mechanisms of cell-to-cell communication exist in different fungi (Lee et al., 2007). However, how yeast cells sense this small peptide remains to be understood. It will be of interest to examine whether the PTR or OPT small-peptide-sensing systems are involved.

GPCRs and Amino Acid Sensing in Fungi

GPCRs have also been reported to sense amino acids in fungi. In *C. albicans*, methionine is important for the yeast-to-hypha transition on solid medium, and Gpr1 has been found to sense methionine to control filamentation in the presence of carbon sources such as glucose (Maidan et al., 2005b). It is presently unclear whether Gpr1 directly senses extracellular methionine, intracellular amino acids, or both.

The GPCR Gpr4 identified in *C. neoformans* was also found to sense amino acids and activate cAMP signaling (Xue et al., 2006). The Gα protein Gpa1 in *C. neoformans* controls cAMP signaling and plays important roles in mating and production of virulence factors, such as capsule formation and melanin production. Gpr4 is a 7-TM protein

that shares structural similarity with the Gpr1 glucose sensor of *S. cerevisiae* and Gpr1 amino acid sensor of *C. albicans*. Mutagenesis and biochemical analyses reveal that Gpr4 directly activates the Gα protein Gpa1 and engages the Gpa1-cAMP signaling pathway. Because Gpr4 contributes to, but is not essential for, the production of virulence factors controlled by cAMP signaling and is not important for melanin production or virulence, additional upstream receptors besides Gpr4 may also contribute to Gpa1-regulated functions. It is also possible that other compounds may contribute to Gpa1 activation, such as glucose-6-phosphate, which has been identified as an important element contributing to PKA signaling in *S. cerevisiae*.

Interestingly, although glucose is a preferred carbon source for *Cryptococcus* and can induce transient cAMP production via the Gpa1 Gα protein, based on direct cAMP assays, Gpr4 is not important for glucose sensing. Similar to Gpr1 in *C. albicans*, Gpr4 has been found to sense amino acids such as methionine, and methionine stimulates mating filament production (Xue et al., 2006). Methionine induces the internalization of a Gpr4-DsRED fusion protein and also induces transient cAMP production in *C. neoformans*, which is blocked by *gpr4* mutations. A low concentration of methionine in the medium stimulates mating-hypha elongation in a Gpr4-dependent manner, but the role of methionine remains to be elucidated at a molecular level in this case. Activation of cAMP signaling by glucose and amino acids represents a nutrient coincidence detection system conserved in other pathogenic fungi. There are Gpr1 and Gpr4 sequence homologs in other fungi that remain to be studied, and it will be of interest to investigate the functions of these membrane proteins.

The Major Nutrient Sensor Tor Is Also Important for Amino Acid Sensing

Tor is a serine/threonine kinase of the phosphatidylinositol kinase-related kinase family, which shares conserved motifs (such as HEAT repeats, FAT, and FATC domains), and is structurally and functionally conserved in eukaryotes (Abraham, 2004). The Tor kinases were first identified in yeast as the targets of the immunosuppressive drug rapamycin, originally isolated from a strain of *Streptomyces hygroscopicus*, and which binds to the FK506-binding protein FKBP12 (Harding et al., 1989; Heitman et al., 1991; Siekierka et al., 1989). *S. cerevisiae* contains two Tor homologs, Tor1 and Tor2, which occur as multiprotein complexes known as TORC1 and TORC2 (Loewith et al., 2002; Wedaman et al., 2003). The TORC1 complex is sensitive to rapamycin and involved in a wide range of functions, with the exception of actin cytoskeleton polarization, which is regulated by the TORC2 complex.

Tor proteins sense nutrient signals, including amino acids, and regulate a broad range of cell developmental and signaling processes, including ribosome biosynthesis, protein translation, starvation-related transcriptional regulation, and autophagy (reviewed by Raught et al., 2001; Rohde et al., 2001; and Rohde and Cardenas, 2003). Rapamycin treatment triggers ubiquitination and degradation of some high-affinity amino acid transporters, such as the tryptophan permease Tat2 and the histidine permease Hip1, suggesting that Tor signaling promotes stability of these high-affinity, specialized transporter systems (Beck et al., 1999; Schmidt et al., 1998). On the other hand, Tor signaling also negatively regulates the stability of general amino acid permease systems such as Gap1 and thus inversely regulates these two classes of amino acid permeases to balance the nutritional requirements of the cell (Beck et al., 1999). A serine/threonine kinase, Npr1, has been recognized as mediating regulation of these permeases by Tor (Raught et al., 2001; Schmidt et al., 1998).

More recently, the Tor pathway was proposed to sense glutamine, a preferred nitrogen source and a key intermediate in yeast nitrogen metabolism. Glutamine depletion in yeast triggers nuclear localization and activation of the TOR-inhibited transcription factors Gln3, Rtg1, and Rtg3 (Crespo et al., 2002). This nitrogen regulation is mediated by the PP2A-like phosphatase Sit4. Many of the nontranscriptional effects of Tor, such as the initiation of translation and control of the stability of amino acid permease, are also regulated via Sit4 (Cardenas et al., 1999; Di Como and Arndt, 1996; Jacinto, 2007; Rohde et al., 2004). Tor-mediated nutrient signaling was recently revealed to trigger nuclear translocation of Gln3, and a role of Golgi body-to-endosome vesicular trafficking in TORC1-controlled nuclear translocation has been described (Puria et al., 2008).

In *S. cerevisiae*, the yeast-to-filament switch in response to nitrogen limitation is controlled by a combination of signaling pathways, including the well-defined MAPK cascade, cAMP-PKA, and Tor signaling (Cutler et al., 2001; Gimeno et al., 1992; Pan and Heitman, 1999). Tor function involves the Sit4 protein phosphatase and is independent from, and parallel to, the MAPK and cAMP-PKA pathways, but it is possible that signaling in response to nutrients involves cross talk between these pathways (Chen and Powers, 2006; Schmelzle et al., 2004; Zurita-Martinez and Cardenas, 2005). Future studies are needed to understand the precise molecular mechanisms by which Tor regulates nutrient sensing and activation.

Sensing Proline and Apoptosis in Fungi

S. cerevisiae can utilize proline when a preferred nitrogen source is not available in the growth medium. Yeast cells import proline through the general amino acid permease Gap1 (low affinity) and the proline-specific permease Put4 (high affinity), which are regulated by nitrogen repression and not induced by proline (Lasko and Brandriss, 1981; Stanbrough and Magasanik, 1995). More recently, two SPS system-regulated permeases (Agp1 and Gnp1) have also been found to transport proline (Andreasson et al., 2004). Two enzymes of the proline utilization pathway, Put1 and Put2, are regulated by a transcriptional activator, Put3, which is a member of the $Zn(II)_2Cys_6$ binuclear cluster protein family and requires a conformational change for activation. Put3 regulates the proline utilization pathway by binding to the promoters of *PUT1* and *PUT2* and turns on the expression of these two genes in the presence of proline and in the absence of preferred nitrogen sources (Des Etages et al., 2001; Huang and Brandriss, 2000). The proline utilization proteins can also be activated by Gal4, another transcriptional activator that belongs to the same gene family as Put3. Gal4 primarily regulates the galactose utilization pathway, suggesting there is cross talk between different nutrient utilization pathways (D'Alessio and Brandriss, 2000).

A recent report on the plant fungal pathogen *Colletotrichum trifolii* showed that this fungus senses proline to trigger apoptosis. Under nutrient starvation, expression of a dominant-active Ras allele leads to elevated levels of ROS, which in turn triggers abnormal fungal growth and an apoptosis-like cell death. Remarkably, this apoptotic response can be rescued by a single amino acid, proline. Further studies suggest proline can function as an efficient antioxidant and

inhibitor of programmed cell death. Proline can also protect wild-type C. *trifolii* cells from various lethal stresses, including UV light, salt, heat, and hydrogen peroxide. The ability of proline to protect fungal cells from ROS-mediated apoptosis may indicate it has a broad role in responding to cellular stress (Chen and Dickman, 2005). It will be of interest to elucidate how, and why, C. *trifolii* senses proline.

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Regulation of Gene Expression by Ambient pH

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Ambient pH regulation of gene expression is a transcriptional regulatory system that enables the presence of gene products appropriate to the pH of the environment and prevents those that are inappropriate to the environmental pH. The genes controlled include those specifying secreted enzymes, permeases and enzymes associated with transport, and enzymes involved in the synthesis of exported metabolites, such as toxins and antibiotics. Much of the progress in elucidating the mechanism of ambient pH gene regulation has been made using *Aspergillus nidulans*, and there are a number of reasons why this organism is particularly favorable for the study of pH regulation. Here we discuss some of these reasons and give a brief description of the current model of fungal pH regulation. For more information, readers can consult a recent review (Peñalva et al., 2008) as well as several earlier reviews (Arst and Peñalva, 2003; Peñalva and Arst, 2002; Peñalva and Arst, 2004).

Mutations affecting pH regulation are highly pleiotropic, so the ability to monitor diverse phenotypes rapidly by use of plate tests enables extensive mutant characterization at a very early stage. The availability of simple colony-staining tests for secreted acid and alkaline phosphatases and the fact that each of these activities is encoded by a single gene (Caddick and Arst, 1986; Dorn, 1965a, 1965b) were crucial to the initial recognition of the pH regulatory system and to its subsequent characterization (Caddick et al., 1986; Peñalva and Arst, 2002; Peñas et al., 2007). Mutations resulting in gene expression appropriate to acidic environments (i.e., acidity-mimicking mutations) confer intense staining for acid phosphatase activity even with growth at alkaline pH but prevent staining for alkaline phosphatase activity. The reverse is true for mutations resulting in gene expression appropriate to alkaline environments (i.e., alkalinity-mimicking mutations), with the proviso that alkaline phosphatase staining is less reliable than acid phosphatase staining. As expected, wild-type colonies stain most strongly for acid phosphatase when grown under acidic conditions and for alkaline phosphatase when grown under alkaline conditions.

Another extremely useful plate test is for utilization of γ-aminobutyrate (GABA) as a nitrogen source in strains lacking the GATA transcription factor AreA and, therefore, unable to utilize nitrogen sources other than ammonium and glutamine (Arst and Cove, 1973; Kudla et al., 1990). The GABA permease gene is expressed much more highly under acidic than under alkaline conditions, and acidity-mimicking mutations allow GABA utilization in the absence of AreA (Arst et al., 1994; Caddick and Arst, 1986; Hutchings et al., 1999). This has been exploited as a selection technique both in haploids (Arst et al., 1994; Caddick and Arst, 1986; Fernández-Martínez et al., 2003; Mingot et al., 1999) and in diploids homozygous for lack of *are*A, where it permits selection of partially dominant mutations (Díez et al., 2002; Vincent et al., 2003) and selection of recessive mutations in diploids additionally heterozygous for an allelic recessive mutation (Calcagno-Pizarelli et al., 2007; Peñas et al., 2007; Tilburn et al., 2005).

Other valuable plate tests include responses to Li^+ and MoO_4^{2-}, to which alkalinity-mimicking mutations confer resistance and acidity-mimicking mutations confer hypersensitivity; to neomycin, to which acidity-mimicking mutations confer resistance and alkalinity-mimicking mutations confer hypersensitivity; to alkaline growth media, on which acidity-mimicking mutants grow poorly or not at all; and to acidic growth media, on which alkalinity-mimicking mutants conidiate poorly or not at all (Caddick et al., 1986; Peñalva and Arst, 2002).

The involvement of the proteasome and multivesicular body (MVB) pathway components in pH regulation (Galindo et al., 2007; Hervás-Aguilar et al., 2007; Peñalva et al., 2008; Vincent et al., 2003; A. M. Calcagno-Pizarelli, A. Hervás-Aguilar, M. A. Peñalva, and H. N. Arst, Jr., unpublished data) has highlighted another very useful feature of *A. nidulans*, the ability to maintain haploid nuclei containing lethal or severely debilitating mutations heterozygously in a heterokaryon. This enables selection of such mutations and investigation of the growth stage at which lethality or severe debilitation results and can permit the possible selection of suppressor mutations allowing sufficient growth for further analysis.

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The presence of the transcription factor PacC (see below) in relatively large amounts in mycelia has enabled very extensive use of electrophoretic mobility shift assays and Western blots to study PacC regulation and processing and ambient pH signal transduction and has facilitated intracellular localization studies (Calcagno-Pizarelli et al., 2007; Denison et al., 1995, 1998; Díez et al., 2002; Espeso et al., 2000; Espeso and Arst, 2000; Fernández-Martínez et al., 2003; Galindo et al., 2007; Hervás-Aguilar et al., 2007; Mingot et al., 1999, 2001; Negrete-Urtasun et al., 1999; Orejas et al., 1995; Peñas et al., 2007; Vincent et al., 2003).

TRANSCRIPTION FACTOR PacC

The *A. nidulans* pH-responsive transcription factor is specified by the *pac*C gene, which is homologous to *Saccharomyces cerevisiae RIM101*. *pac*C potentially encodes 678 residues; however, translation initiates at codon 5 to give a 674-residue translation product with a predicted mass of 72 kDa and accordingly denoted $PacC^{72}$ (Mingot et al., 1999; Peñalva and Arst, 2002). Features of PacC are schematized in Fig. 1.

PacC Is a Cys_2His_2 Zinc Finger Protein

The PacC DNA binding domain (DBD) located towards the amino terminus comprises three Cys_2His_2 zinc fingers, which are highly conserved in interspecies alignments with PacC/Rim101 homologues. The sequence within the PacC DBD, particularly the C-terminal finger, and the occurrence of an unusually long linker between PacC fingers 1 and 2 also resemble the DBDs of members of the GLI (glioma-associated protein) family of transcription factors, which includes the metazoan GLI homologue *Drosophila melanogaster* Cubitus interruptus (Ci) (Espeso et al., 1997; Pavletich and Pabo, 1993; Tilburn et al., 1995). Sequence similarity and modeling predict that fingers 2 and 3 mediate specific DNA binding to GCCARG consensus sites in PacC-regulated gene promoters, whereas the first finger is not thought to contact the DNA directly but to stabilize the interactions of finger 2 (Espeso et al., 1997).

PacC Is Activated by Two-Step Proteolysis

Under strongly acidic growth conditions in the absence of ambient pH signaling, the $PacC^{72}$ protein predominates in a closed conformation held together by interactions involving three regions, denoted regions A, B, and C (Espeso et al.,

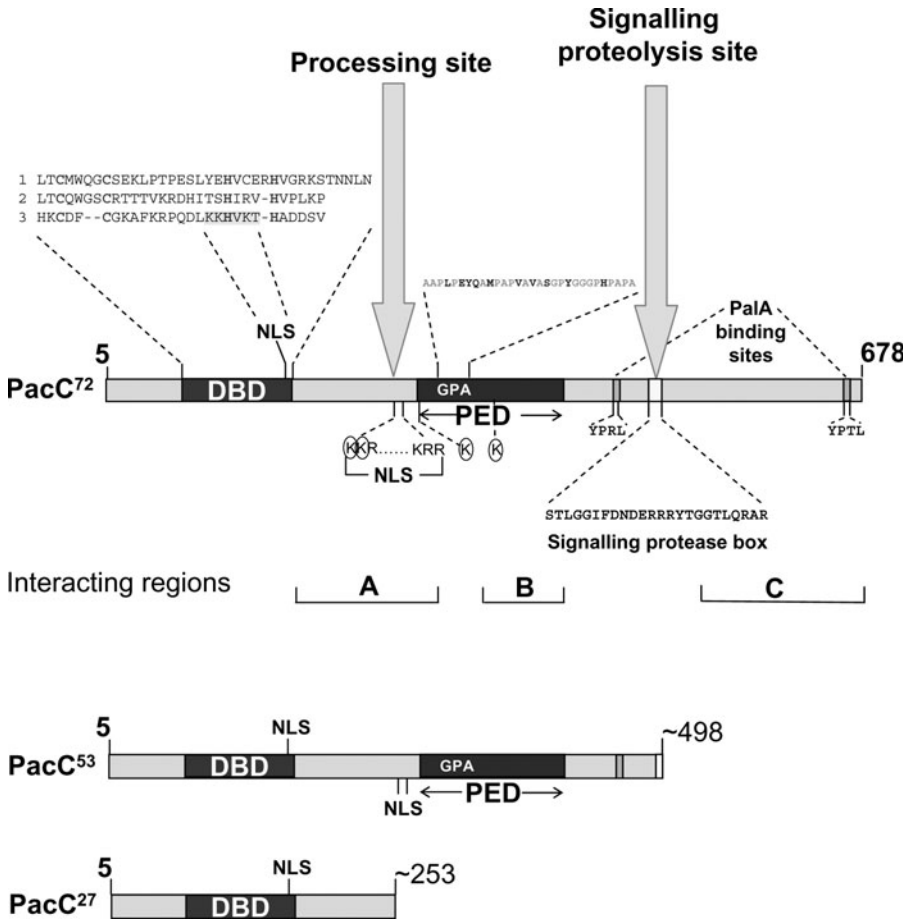

FIGURE 1 Schematic representation of PacC features. GPA, Gly-Pro-Ala rich region; PED, processing efficiency determinant. The sequences of the three Cys_2His_2 zinc fingers are shown above the scheme; zinc-chelating residues are shown in bold, and the putative monopartite NLS is shaded. The putative bipartite NLS is shown. Circled Ks indicate the Lys252, Lys253, Lys267, and Lys337 residues, whose simultaneous substitution by Arg residues impairs PacC processing.

2000). On exposure to neutral-to-alkaline conditions and in response to a signal mediated by the Pal signal transduction pathway, PacC undergoes a limited two-step proteolysis (Díez et al., 2002).

Signaling Proteolysis

The first step, signaling proteolysis, is the pH-regulated step that results in truncation of PacC between residues 493 and 500 within the 24-residue highly conserved signaling protease box, removing some ~180 C-terminal residues which include a negative-acting C terminus containing interacting region C, to form $PacC^{53}$ (Díez et al., 2002; Espeso et al., 2000). Two YPXL/I protein-protein binding motifs, which are recognized by signal transduction component PalA (*S. cerevisiae* Rim20p), flank the PacC signaling proteolysis box, and it is thought that PalA PacC binding is a prerequisite for the signaling proteolysis (Vincent et al., 2003; see also below). Signal transduction component PalB and the yeast homologue Rim13 (also known as Cpl1p) are predicted calpain-like cysteine proteases (Denison et al.,1995; Futai et al., 2001; Li et al., 2004; Sorimachi et al., 1997; Xu and Mitchell, 2001). PalB is almost certainly the signaling protease; it is the only predicted protease in the pH signal transduction pathway, and it has been shown to be essential for the signaling proteolysis but not to be required for the subsequent processing step (Peñas et al., 2007). The calpain-like nature of PalB is supported by sequence and mutational analysis. Furthermore, scanning mutagenesis of the PacC signaling protease box has demonstrated that, with the exception of Leu498, where mutation results in processing recalcitrance, sequence determinants in the region of the signaling proteolytic cleavage are not stringent (Díez et al., 2002; Peñas et al., 2007). As calpains recognize bonds between regulatory domains rather than specific sequences and cleave proteins in a limited manner (reviewed by Suzuki et al., 2004), PalB is ideally suited to the task of removing the negatively acting PacC C terminus. The *A. nidulans* signaling proteolysis step corresponds to the single cleavage event in the proteolytic activation of *S. cerevisiae* Rim101 (Li and Mitchell, 1997; Xu and Mitchell, 2001).

Processing Proteolysis

The product of the signaling proteolysis, $PacC^{53}$, is thought to be accessible to the processing protease due to an open conformation assumed in the absence of interacting region C, and the processing proteolysis occurs in a pH-independent manner, removing a further ~250 C-terminal residues to form $PacC^{27}$ (Díez et al., 2002; Galindo et al., 2007; Hervás-Aguilar et al., 2007; Mingot et al., 1999; Orejas et al., 1995; Peñas et al., 2007). $PacC^{27}$ is an activator of alkaline-expressed genes, such as *ipn*A, encoding isopenicillin-N synthase (Espeso and Peñalva, 1996), and a repressor of acid-expressed genes such as *gab*A, encoding the major GABA permease (Espeso and Arst, 2000).

The processing proteolysis is almost certainly mediated by the proteasome. This has been strongly suggested by several pieces of evidence. First, despite the availability of the powerful GABA selection technique described above, it has not been possible to obtain mutations in the processing protease, suggesting that such mutations result in lethality, consistent with the essential function of the proteasome. Second, if the requirement for the signaling proteolysis is bypassed, PacC is processed in *S. cerevisiae*, which demonstrates that the processing protease is conserved in yeast, even though Rim101p is not itself a substrate. Third, PacC processing resembles that of the proteasomal activation of other transcription factors, particularly that of NF-κB and *Drosophila* Ci (Tian et al., 2005), in a number of respects. (i) Processing is independent of sequence at the processing site, and the site of cleavage is determined by remote sequence or structure (Mingot et al., 1999). (ii) A processing efficiency determinant has been discovered C terminal to the processing site, thought to be exposed following the signaling proteolysis (Díez et al., 2002; Mingot et al., 1999). (iii) There is a tightly folded domain, a potential impediment to the unfolding required for proteasomal progression (Tian et al., 2005) in the direction of proteasome movement in the form of the N-terminal zinc finger region, which resembles that of Ci in sequence and predicted structure (Espeso et al., 1997).

Relatively recent studies by Hervás-Aguilar et al. (2007) have demonstrated that the processed PacC C terminus is heterogeneous, in agreement with the model proposed for proteasomal cleavage proposed by Tian et al. (2005). Moreover, $preB2^{K101R}$, a viable mutation created by reverse genetics in the active site of proteasome subunit β5, and the corresponding $pre2^{K108R}$ in yeast change the scissile bonds cleaved when PacC is processed in *A. nidulans* and *S. cerevisiae*, respectively. This indicates that PacC is a direct proteasomal substrate, which is strongly supported by the further finding that $preB2^{K101R}$ suppresses a deletion of the processing efficiency determinant (PED) for $PacC^{27}$ production. In addition, Arg substitutions of four potentially ubiquitinable Lys residues, within or near the PED impair processing, and deletion of a Gly-Pro-Ala rich region, a candidate low-complexity sequence favoring processing rather than destruction by the proteasome, destabilize PacC.

A small amount of $PacC^{27}$ is formed in the absence of Pal signaling. This is thought to be due to traces of $PacC^{72}$ in an open-processing protease-accessible conformation in equilibrium with the majority of $PacC^{72}$ in the closed processing protease inaccessible conformation (see Fig. 3).

PacC Localization

Under acidic conditions in the absence of Pal signaling, where $PacC^{72}$ predominates, PacC is largely, but not exclusively, cytoplasmic. Under alkaline conditions in the presence of a functional signal transduction pathway where $PacC^{27}$ predominates, PacC is largely nuclear and, in addition, $PacC^{53}$ appears to be nuclearly localized (Mingot et al., 2001). $PacC^{72}$ and $PacC^{53}$ contain a bipartite nuclear localization signal (NLS) between residues 252 and 269, which is absent in $PacC^{27}$ (Tilburn et al., 1995) (Fig. 1). It is thought that this NLS may participate in the nuclear localization of $PacC^{53}$ (Fernández-Martínez et al., 2003; Mingot et al., 2001) and possibly also in the nuclear localization of $PacC^{72}$. Deletion analysis has shown that finger 3 in the DBD suffices to localize green fluorescent protein to the nucleus, and further mutational analysis and interspecies alignments have demonstrated that it contains, within a highly conserved region, a putative basic NLS, or part thereof, which resembles a classical monopartite NLS (Fernández-Martínez et al., 2003; Jans et al., 2000; Mingot et al., 2001) (Fig. 1).

THE pH SIGNALING PATHWAY

The pH Signaling Pathway Proteins

In *A. nidulans* and in unicellular yeasts, the pH signaling pathway involves six dedicated proteins (seven in *S. cerevisiae*). Significant advances in our current understanding of pH signaling have also come from research using budding yeast as experimental model (Peñalva et al., 2008). The *A. nidulans* protein nomenclature and that of the corresponding orthologues in *S. cerevisiae* are shown in Fig. 2.

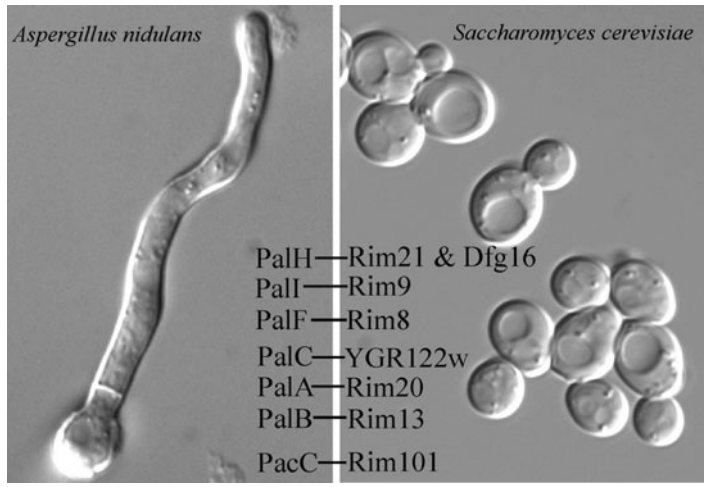

FIGURE 2 pH signal transduction pathway proteins: nomenclature of dedicated pH signal transduction pathway proteins in *A. nidulans* and *S. cerevisiae*.

PalH contains seven transmembrane (TM) domains (Calcagno-Pizarelli et al., 2007; Negrete-Urtasun et al., 1999) and has two homologues in *S. cerevisiae*, Rim21 and Dfg16 (Barwell et al., 2005). PalI contains three TM domains (Calcagno-Pizarelli et al., 2007; Denison et al., 1998), and its Rim9 *S. cerevisiae* homologue lacks the long PalI hydrophilic tail (Li and Mitchell, 1997). PalF (Rim8 in *S. cerevisiae*) contains arrestin N-terminal and C-terminal domains characterizing mammalian β-arrestins (Herranz et al., 2005; Maccheroni et al., 1997). PalA and PalC have Bro1 domains (Kim et al., 2005) and bind the endosomal sorting complex required for transport III (ESCRT-III) component Vps32 (Galindo et al., 2007; Negrete-Urtasun et al., 1997; Tilburn et al., 2005; Vincent et al., 2003; Xu and Mitchell, 2001). Rim20 (Xu and Mitchell, 2001) and YGR122w (Barwell et al., 2005; Galindo et al., 2007; Rothfels et al., 2005) are the PalA and PalC *S. cerevisiae* orthologues, respectively. The presence of a PalC orthologue in yeasts has been recently confirmed by work in *Yarrowia lipolytica* (Blanchin-Roland et al., 2008). Finally, as noted above, PalB and its yeast orthologue Rim13 are calpain-like cysteine proteases that contain the catalytic domain of calpains but lack the Ca^{2+}-sensing domain of these regulatory proteases (Denison et al., 1995; Futai et al., 1999; Li et al., 2004; Xu and Mitchell, 2001).

Many of these pH-signaling proteins have relatively close mammalian homologues. While this has been discussed in recent reviews (Arst and Peñalva, 2003; Peñalva et al., 2008; Peñalva and Arst, 2004), it is worth mentioning here that the pH signaling pathway shares remarkable similarity with the mammalian Hedgehog pathway, which involves a 7-TM domain receptor, an accessory TM domain-containing protein, an arrestin, and the signal-regulated, proteasome-mediated proteolytic processing of a zinc finger transcription factor (Ci in *D. melanogaster*) to control its subcellular localization (Arst and Peñalva, 2003; Peñalva et al., 2008).

The mammalian orthologue of PalB is calpain 7 (Futai et al., 2001). The most likely mammalian orthologue of PalA is Alix, a protein that plays multiple roles in membrane traffic-related processes (Odorizzi, 2006). PalA is notable in that it has a paralogue denoted BroA. *S. cerevisiae* Bro1 plays an important role in the MVB pathway but is not involved in pH signaling (Luhtala and Odorizzi, 2004; Nikko et al., 2003; Nikko and Andre, 2007; Odorizzi et al., 2003; Springael et al., 2002; an extensive discussion may be found in Peñalva and Arst, 2004). A recent report strongly indicates that, in mammalian cells, the Bro1 domain- and His domain-containing phosphotyrosine phosphatase type N23 (and not Alix) plays the MVB pathway roles of fungal Bro1 (Doyotte et al., 2008).

The Subcellular Organization of the pH Signaling Pathway

Work in *A. nidulans* and *S. cerevisiae* strongly supports the model shown in Fig. 3, in which membrane trafficking is intimately associated with pH signaling. One key aspect of this model is that the pH signaling pathway represents one example of positive-acting association between endocytosis and signaling. This is notable because endocytosis, which is usually associated with membrane protein degradation/receptor downregulation, generally plays a negative role in signal transduction. Receptor downregulation by endocytosis occurs because membrane proteins that follow the endocytic pathway all the way down to the vacuole are sorted into vesicles that bud inside the prevacuolar endosome lumen to form multivesicular endosomes. These MVBs fuse with vacuoles, where luminal vesicle-associated proteins are degraded by vacuolar hydrolases (Hierro et al., 2004; Hurley and Emr, 2006; Katzmann et al., 2002). However, if a receptor reaching the endosomal system from the plasma membrane avoids being sorted into MVB vesicles, it can either signal from endosomes or recycle back to the plasma membrane for new rounds of signal reception.

The proteins of the pH signaling pathway are organized in two spatially separated complexes. The plasma membrane pH signaling complex contains PalH, PalI, and PalF. The 7-TM domain protein PalH is almost certainly the receptor in the pathway (Calcagno-Pizarelli et al., 2007). PalI is not absolutely essential for pH signaling, since unlike null mutations in any other pH signaling gene, null mutations in *palI* allow some growth at alkaline pH. It has been shown that PalI is a 3-TM helper protein assisting the plasma membrane localization of PalH, although it may play additional roles in signaling (Calcagno-Pizarelli et al., 2007). PalF is an arrestin-like protein. Mammalian arrestins interact with the cytosolic tails of 7-TM receptors. PalF interacts strongly with the ~400-residue cytosolic tail of

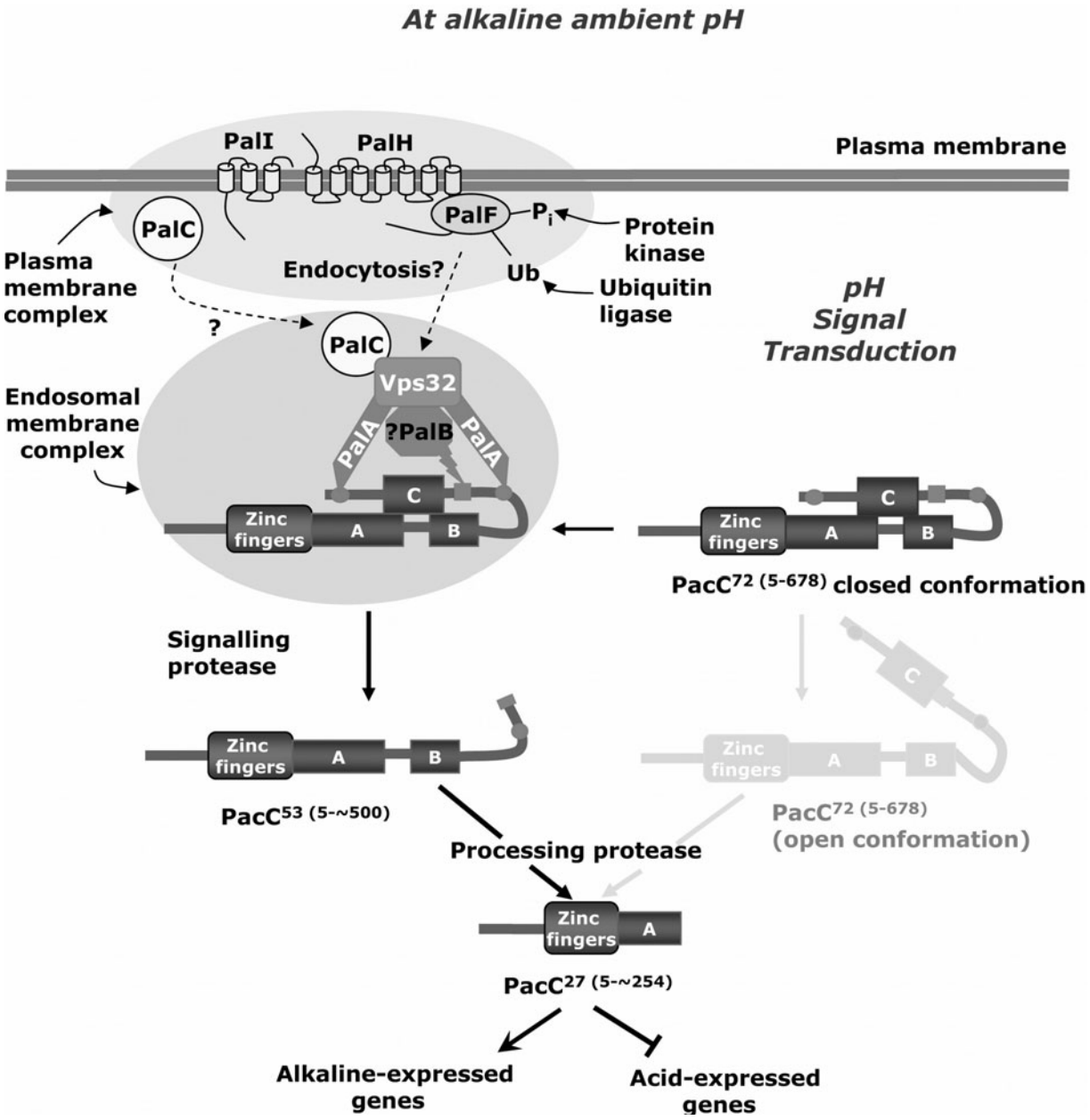

FIGURE 3 Summary of ambient alkaline pH signaling and PacC processing. The known or hypothesized locations of the six dedicated Pal proteins of the pH signal transduction pathway are indicated along with those of the ESCRT-III protein Vps32 and the PacC transcription factor in the two complexes involved in the signal transduction process. Interacting regions A, B, and C of PacC are shown as rectangles, the signaling protease box is indicated by a small square, and the PalA-binding YPXL/I motifs are shown as small circles. Conversion of full-length $PacC^{72}$ to $PacC^{53}$ by the signaling protease (PalB) and on to $PacC^{27}$ by the processing protease (the proteasome) is shown. Although the great majority of $PacC^{72}$ is in the closed, inaccessible conformation, trace amounts are apparently in an open conformation, accessible to the processing protease, and can be converted directly to $PacC^{27}$ without the participation of pH signal transduction (indicated in pale gray). (Reproduced from Peñalva et al., 2008, with permission, having been updated and modified from Peñas et al., 2007.)

PalH, and this interaction is crucial for pH signaling (Herranz et al., 2005). Resembling mammalian arrestin/7-TM combinations, exposure of cells to alkaline pH results in the multiple ubiquitination of PalF in a PalH-dependent manner. As multiple ubiquitination is a demonstrated signal for the sorting of plasma membrane receptors into endocytic vesicles and as mammalian arrestin ubiquitination promotes the endocytic internalization of the cognate 7-TM receptor (DeWire et al., 2007; Lefkowitz and Shenoy, 2005), these findings indicate that one role of PalF would be promoting the endocytic internalization of PalH and that such internalization is required for pH signaling.

Compelling evidence obtained in studies of *S. cerevisiae* and *A. nidulans* strongly supports the existence of a second pH signaling complex associated to the ESCRT complexes on endosomal membranes. For membrane proteins trafficking through the endosomal system, sorting into inwardly budding vesicles that give rise to MVBs represents an unavoidable death verdict. Thus, it should come as no surprise that this step is highly regulated. Protein sorting and inward vesicle budding involve the action of four heteromeric protein complexes, denoted ESCRT-0, I, II, and III, which are thought to act sequentially in the so-called MVB pathway (Hurley and Emr, 2006; Katzmann et al., 2002; Williams and Urbe, 2007). ESCRT-III is made of four proteins denoted Vps2, Vps24, Vps20, and Vps32. While it is known that these four proteins are organized into two different Vps2/Vps24 and Vps20/Vps32 subcomplexes (Babst et al., 2002), their actual stoichiometry in the complex is as yet unknown, although current models assume that ESCRT-III proteins form a lattice on endosomal membranes. This lattice would provide a platform on which the second pH signaling complex would assemble (Hurley and Emr, 2006; Williams and Urbe, 2007).

The first indication that the pH signaling pathway involves endosomal membrane ESCRT complexes came from work with *A. nidulans* PalA and *S. cerevisiae* Rim20, which contain a prototypical Bro1 domain (Negrete-Urtasun et al., 1997; Xu and Mitchell, 2001). This domain is used by the MVB pathway regulatory factor Bro1 to bind ESCRT-III Vps32 (Kim et al., 2005), and PalA and Rim20 do indeed bind Vps32 (Boysen and Mitchell, 2006; Vincent et al., 2003; Xu and Mitchell, 2001), and *S. cerevisiae* Rim20 localizes to endosomes in a pH- and Vps32-dependent manner (Boysen and Mitchell, 2006). In addition, PalA and Rim20 bind a tetrapeptidic motif whose consensus is YPXI/L, where X indicates any amino acid (Vincent et al., 2003). Two such motifs flank the signaling protease cleavage site in PacC/Rim101, and this binding is required for pH signaling (Vincent et al., 2003). Thus, PalA/Rim20 not only bind endosomal membrane complexes (see also below) but can also recruit PacC/Rim101 to these complexes. Rim13 and PalB are components of this endosomal pH signaling complex. Rim13 also interacts with Vps32 (Ito et al., 2001), whereas PalB could potentially be recruited to ESCRT-III through its N-terminal MIT domain. It has been suggested that one additional role of Rim20 binding to YPXL motifs flanking the signaling protease site is contributing to cleavage specificity by appropriately positioning the Rim13 signaling protease (Xu and Mitchell, 2001).

Work in yeasts demonstrated that all components of ESCRT-I, ESCRT-II, and the ESCRT-III Vps32/Vps20 subcomplex are required for pH signaling (Blanchin-Roland et al., 2005; Cornet et al., 2005; Hayashi et al., 2005; Kulas et al., 2004; Rothfels et al., 2005; Xu et al., 2004). Notably, deletion of ESCRT-III Vps2 or Vps24 and of the ESCRT-disassembling AAA ATPase Vps4 results in partially constitutive signaling (Hayashi et al., 2005). This demonstrates that the MVB pathway function of ESCRT-III is not required for pH signaling and supports the view that the pH signaling role of Vps32/Vps20 in the ESCRT-III lattice is acting as anchoring platforms on which the endosomal pH signaling complexes assemble.

The ambient alkaline pH and PalH-dependent ubiquitination of PalF is arguably the most reliable indicator of ambient pH signaling pathway activation. PalF ubiquitination is independent of PalA, PalB, and PalC and, as expected from the *palI* null phenotype/accessory role, partially dependent on PalI (Herranz et al., 2005). This, together with epistasis analyses in *S. cerevisiae* (Hayashi et al., 2005), strongly supports the current model in which the plasma membrane complex acts upstream of the endosomal membrane complex (Fig. 3). The latter contains the PalB protease and can recruit, at least transiently, PacC to catalyze its signaling protease cleavage.

The Spatial Segregation of the Two Ambient pH Signaling Complexes: Filling the Gap

The two pH signaling complexes are spatially segregated. Seminal work by the Mitchell laboratory demonstrated that the endosomal complex component Rim20 indeed localizes to endosomes (Boysen and Mitchell, 2006), whereas PalH and PalI are found on the plasma membrane (Calcagno-Pizarelli et al., 2007). Thus, pH signaling must involve a mechanism mediating communication between the two complexes. One candidate to mediate this communication is PalC, until recently the most enigmatic of the *pal* signaling proteins. PalC contains a Bro1-like domain (Tilburn et al., 2005) and binds Vps32 (Galindo et al., 2007). However, PalC is not observed on endosomes but instead localizes to discrete punctate structures associated with the plasma membrane and does so in an ambient pH- and PalH-dependent but PalA-independent manner. Therefore, PalC acts downstream of PalH, upstream of, or in concert with, the endosome signaling complex, can be recruited to the plasma membrane, and has additionally the ability to bind to ESCRT-III, thereby fulfilling the features expected for a bridging protein (Galindo et al., 2007).

The finding that the PalF arrestin plays a positive role in pH signaling and that it is multiply ubiquitinated in an alkaline-pH-dependent manner strongly supports the view that pH signaling involves traffic of plasma membrane PalF-bound PalH to the endosome. PalH endocytosis would be required for pH signaling possibly by facilitating the activation of the PacC signaling protease cleavage-catalyzing complex on endosomal membranes. In this model, PalC would be a bivalent adaptor that would be recruited to the PalH/PalF complex and would facilitate the connection of this complex with the endosomal complex through its binding to Vps32. Thus, the pH signaling pathway represents a paradigmatic example of vesicle-associated signal transduction (Mitchell, 2008).

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Heat Shock Response

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All organisms display a dramatic response to high-temperature stress, in which they massively alter gene expression to synthesize a distinct group of proteins (Parsell and Lindquist, 1993). Although first observed in the polytene chromosomes of *Drosophila* larvae, the regulation of this response and the functions of the synthesized heat shock proteins (hsps) have been particularly well elucidated in *Saccharomyces cerevisiae*, in part through the use of directed mutagenesis (Plesofsky, 2004). When cells are exposed to a high but sublethal temperature, their biosynthesis shifts transiently to produce large quantities of hsps that help cells survive subsequent exposure to otherwise lethal temperatures and other stresses. It was later determined that these hsps are chaperones that minimize the aggregation of other denatured cellular proteins and help restore them to their native conformation (Hartl, 1996). Rather than novel proteins, most hsps turned out to be normal cellular constituents, many of them essential proteins, whose synthesis increased under heat stress. Increased synthesis of these hsps was also seen in response to other physical and chemical stresses and during developmental transitions. These chaperone hsps are not the only proteins induced by heat shock. Enzymes involved in glycolysis, trehalose synthesis, and proteolysis are also induced. Other stresses, particularly oxidative stress and osmotic stress, also elicit characteristic changes in gene expression that overlap with one another and with heat stress. The same regulatory factors may be involved in multiple stress responses. These include the heat shock transcription factor and the stress mitogen-activated protein kinases (MAPKs) Hog1 and Slt2 of *S. cerevisiae* and their orthologs in other organisms.

HEAT SHOCK-INDUCED PROTEINS ACTIVE AS CHAPERONES

Hsp70

Hsp70, related to the DnaK protein of *Escherichia coli* (50% homology), is strongly induced by heat shock (Plesofsky, 2004). This family of proteins (Table 1) function as chaperones that facilitate the native folding of protein substrates and prevent their aggregation. Hsp70 has a well-conserved amino-terminal ATP-binding domain, a less conserved carboxy-proximal domain that binds peptides, and a variable carboxy-terminal tail. ATP hydrolysis is coupled to the release and unfolding of the bound peptide. This ATPase activity is enhanced by the cochaperone J proteins (below), and nucleotide exchange of ADP for ATP is facilitated by Sse cochaperones (below) and Bag domain proteins. There are multiple Hsp70s in any organism, only some of which are induced by heat shock. Functional groups have been determined in *S. cerevisiae* by complementation analysis, although members of a group may differ in inducing stimulus. Among the cytosolic Hsp70 proteins of yeast, the Ssa group contains the principal heat-induced hsps, as well as constitutive proteins, and at least one Ssa is required for growth at normal temperature and for sustained growth at elevated temperature. The Ssa proteins participate in the import of proteins from the cytosol into the mitochondria and the endoplasmic reticulum (ER), and they have been identified as members of DNA checkpoint complexes. Ssa proteins have also been found to be cell wall components. The Ssb group of Hsp70s, in contrast, are repressed by heat shock and are required for growth at low temperature and for cation homeostasis. The Ssb proteins are in a complex with Ssz1, another Hsp70-related protein, that associates with active ribosomes and nascent peptides, and they cooperate in enhancing translational fidelity and proper protein folding. Ssz1 is unusual in that neither its ATPase nor its peptide-binding domain is essential for protein function.

There are four members of the Ssa group and two members of the Ssb group in *S. cerevisiae*, but *Neurospora crassa* encodes only one member of each group, as well as Ssz1 (Table 1). In addition, the *N. crassa* genome encodes three Hsp70-related proteins that do not resemble specific yeast proteins (Borkovich et al., 2004). Expression of both the dominant Ssa-related Hsp70 (F. Squina and A. Rossi, personal communication) and the Ssc-related mitochondrial Hsp70 (below) is increased in *N. crassa* by exposure to acid pH (Leal et al., 2009).

In addition to the cytosolic Hsp70 members, *S. cerevisiae* has three Hsp70s that localize to the mitochondrial matrix

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TABLE 1 Major Hsps of *S. cerevisiae* and homologs in *N. crassa*

HSP	Location	*N. crassa* gene no.	% Identity
Hsp70			
Ssa	Cytoplasm/nucleus	NCU09602	78
Sse	Cytoplasm	NCU05269	48
Ssb	Cytoplasm/ribosomes	NCU02075	64
Ssz1	Cytoplasm/ribosomes	NCU00692	45
Ssc	Mitochondria	NCU08693	71
Grp78	ER	NCU03982	75
Lhs1	ER	NCU09485	26
Hsp90	Cytoplasm/Nucleus	NCU04142	73
Hsp60	Mitochondria	NCU01589	75
Clp			
Hsp104	Cytoplasm	NCU00104	49
Hsp78	Mitochondria	NCU03982	57
Hsp40			
Ydj1	Cytoplasm	NCU07414	50
		NCU00465	38
Djp1	Cytoplasm	NCU06052	39
Sis1	Cytoplasm	NCU03732	43
Jjj3	Cytoplasm	NCU02805	28
Jjj1	Cytoplasm/ribosomes	NCU02432	36
Zuo1	Cytoplasm/ribosomes	NCU03009	47
Mdj1	Mitochondria	NCU05196	39
Jac1	Mitochondria	NCU07360	31
Pam18	Mitochondria	NCU00075	66
Scj1	ER	NCU11102	39
Sec63	ER	NCU00169	29
Hlj1	ER	NCU03335	39

and two that are in the ER. The mitochondrial Ssc1 and Ecm10 are highly homologous, with 82% identity, yet Ssc1 is an essential protein required for protein import and folding, whose loss cannot be compensated by high-level expression of Ecm10. Ssc1 is an integral part of the Tim44 complex and is a necessary component of the protein import motor, binding the preprotein and causing its unfolding and translocation. Ssc1 also functions as a subunit of the endonuclease Endo.Sce1, which likely contributes to recombination of mitochondrial DNA. *N. crassa* encodes one protein with high homology to Ssc1 (71%) and Ecm10 (68%). This Hsp70 also plays a central role in protein import into mitochondria. The third yeast mitochondrial Hsp70 is Ssq1, which mediates the assembly of mitochondrial proteins containing iron/sulfur clusters. In the absence of Ssq1, yeast growth was retarded at low temperatures and intracellular iron accumulated. There is no obvious homolog of Ssq1 in *N. crassa*.

BiP/Kar2/Grp78 is an essential protein in yeast and is the major ER-localized Hsp70. It binds to precursors of transmembrane and secretory proteins, thereby stabilizing these partially unfolded proteins. Grp78 aids in protein import into the ER and is required for membrane fusion during karyogamy. In yeast and *N. crassa*, it is induced to high levels by conditions that increase the accumulation of unfolded proteins, such as heat shock and inhibitors of glycosylation. Grp78 has an ER retention signal at its carboxy terminus. Lhs1/Grp170 is a noncanonical Hsp70 of yeast that is also localized in the ER; it shares only 24% identity with Grp78 and is not essential. Although Lhs1 is not induced by high temperature, it is required to prevent heat-induced aggregation of microsomal proteins. Grp78 and Lhs1 interact to enhance one another's ATP hydrolysis and exchange activities (Steel et al., 2004), and they are both upregulated in the unfolded protein response. The genome of *N. crassa* encodes an Lhs1/Grp170 homolog (26% identity) that has been identified in folding complexes of the ER (Tremmel et al., 2007).

Sse1 and Sse2 are *S. cerevisiae* homologs of mammalian Hsp110, a subgroup of Hsp70, with which it shares 30% identity. The fungal homologs, including *N. crassa* HSP88 and *Schizosaccharomyces pombe* Pss1, which show 45 to 50% identity to one another, are all induced by heat stress, and Pss1 is also induced by nitrogen starvation. Loss of Sse1 leads to slow growth and temperature sensitivity. The Sse proteins were initially identified by their binding to Ca^{2+}-calmodulin. The most important function of Hsp110 appears to be its binding to denatured peptides, which can be refolded only in the presence of Hsp70, with which it forms a complex. Sse1 binds to either Ssa or Ssb Hsp70s. It stimulates their ATPase activity synergistically with cochaperone Ydj1 (Shaner et al., 2005), and it enhances their nucleotide exchange of bound ADP following ATP hydrolysis (Dragovic et al., 2006). Sse1 also functionally interacts with Hsp90 complexes. HSP88 of *N. crassa* binds to its major small hsp, HSP30.

J Proteins

The J proteins or Hsp40s (Table 1) are cochaperones of Hsp70s, just as its founding member *E. coli* DnaJ assists DnaK. At a minimum, these proteins contain a J domain that binds to the ATP-binding domain of Hsp70 and enhances its intrinsically weak ATPase activity. Based on sequence homology to DnaJ, there are three classes of J proteins in *S. cerevisiae*. Class I, with the highest sequence homology, contains an N-terminal J domain, a glycine/phenylalanine (G/F)-rich region, a central cysteine-rich zinc finger domain, and a C-terminal peptide-binding domain. Class II proteins have the J domain and G/F-rich region, and class III members have only the J domain. Of the 22 encoded J proteins (Walsh et al., 2004), five are class I, four are class II, and 13 are class III. Three class I proteins (Ydj1, Xdj1, and Apj1) are localized in the cytoplasm, one in the mitochondria (Mdj1), and one in the ER (Scj1). There are two class II proteins in the cytoplasm (Sis1 and Djp1), one in the ER (Hlj1), and one in the nucleus (Caj1). Of the class III proteins, five are cytoplasmic (Zuo1, Swa2, Jjj1, Jjj2, and Jjj3), four are mitochondrial (Mdj2, Pam18, Jac1, and Jid1), three are in the ER (Jem1, Sec63, and Erj5), and one is nuclear (Cwc23). Three of the mitochondrial and two of the ER class III proteins have transmembrane domains, as does one class II ER protein.

Clearly, there are more J proteins than Hsp70s, which may have multiple J protein partners. Ydj1 (Mas5) is the most abundant of the cytosolic J proteins, followed by Zuo1, Sis1, and Djp1 (Sahi and Craig, 2007). Both Ydj1 and Sis1 are induced by high temperature, but Sis1 is an essential protein due to its unique G/F region. Ydj1 is a cochaperone of the Ssa Hsp70s, assisting in protein import into mitochondria and the ER. The slow growth evinced by yeast cells in which *YDJ1* has been deleted can be rescued by the J domains of various cytosolic J proteins (Sahi and Craig, 2007). The essential Sis1 is required for mRNA translation initiation and tends to concentrate at nuclei. Zuo1 assists the Ssb group of Hsp70s and Ssz1 and, like them, is associated with active ribosomes and nascent peptides. Deletion of *ZUO1* causes sensitivity to cold and to cations. Djp1 is required for protein import into peroxisomes.

Mdj1 in the mitochondrial matrix cooperates with Hsp70 Ssc1 in the folding of mitochondrially synthesized and imported proteins. Cells in which *MDJ1* is deleted cannot grow on respiratory substrates at normal temperature or on any substrate at high temperature. Mdj2 is integral to the inner mitochondrial membrane and has overlapping functions with Mdj1. Pam18 also cooperates with Ssc1 but as a component of the mitochondrial protein import motor. Jac1 is an essential protein that cooperates with Ssq1 Hsp70 to assemble Fe/S complexes in the mitochondrial matrix. In the lumen of the ER, several J proteins cochaperone the Grp78 Hsp70. Scj1 cooperates with Grp78 in protein folding and maturation, and transmembrane Jem1 assists Grp78 in nuclear membrane fusion during mating. Both these J proteins cochaperone Grp78 in ER-associated degradation of proteins (Nishikawa et al., 2001), and codisruption of *SCJ1* and *JEM1* caused growth arrest at high temperature. Sec63, also integral to the ER membrane, is an essential protein that is required for translocation of proteins into the ER, in cooperation with Grp78. On the other hand, transmembrane Hlj1 cooperates with cytosolic Ssa Hsp70s in ER-associated degradation of membrane proteins (Huyer et al., 2004).

Several encoded proteins with J domains have been identified in other fungal genomes (see the Broad Institute website [http://www.broadinstitute.org/]). Since the *N. crassa* genome has 22 of these predicted proteins, many with homology to specific yeast proteins (Table 1), it is likely that most of the J proteins of yeast are also made by *N. crassa*. The Ssb Hsp70 and Zuo1 homologs of *N. crassa* were found to be upregulated early in conidial germination, in association with ribosomal biogenesis (Kasuga et al., 2005), and upregulation of Ssa Hsp70 and the Sis1-like J protein was induced by double-stranded RNA (Choudhary et al., 2007).

Hsp90

There are two nearly identical (97%) genes for *S. cerevisiae* Hsp90, an abundant protein that has widespread cellular effects (Plesofsky, 2004). *HSC82* is expressed more strongly during normal growth, and *HSP82* is induced by heat shock. Although the two Hsp90s can substitute for one another, two genes are required for growth at high temperature, while one is required for cell viability. Dimeric Hsp90 is a chaperone that folds other proteins through binding and hydrolyzing ATP. In addition to aiding protein recovery from stress, Hsp90 regulates many key cellular proteins, including protein kinases, transcription factors, and hormone receptors. There are several cochaperones for Hsp90 that either enhance or diminish its ATP-hydrolyzing activity. A common attribute of these cochaperones is that they bind to a C-terminal pentapeptide (MEEVD) of Hsp90 by means of tetratricopeptide repeats (TPRs). These cochaperones can also bind a similar C-terminal sequence in the major chaperones, Hsp70 and Hsp104 (below), via TPRs. Sti1 has two TPR domains, the N-terminal of which can interact with Hsp70 or Hsp104, while the C-terminal domain interacts with Hsp90. In this way, Sti1, which is induced by heat shock, functions as a chaperone adaptor protein.

Hsp90 is important for regulating yeast cell cycle progression into mitosis, with lowered levels leading to cell cycle arrest in G_2 at 37°C; this effect is modulated by the Sch9 protein kinase. In *S. pombe*, Hsp90 was found to stabilize Wee1 tyrosine kinase, which negatively regulates entry into mitosis. The pheromone signaling pathway of *S. cerevisiae* requires Hsp90 through its interaction with Ste11 of a MAPK signaling cascade. Furthermore, a single amino acid deletion in Hsp90 suppressed vegetative incompatibility in *Podospora anserina* and altered sexual spore formation. In *S. pombe*, Hsp90 was required for protein kinase A (PKA)-mediated glucose repression of genes involved in gluconeogenesis and sexual development (Alaamery and Hoffman, 2008).

Another protein kinase regulated by Hsp90 is Gcn2 of *S. cerevisiae*. This kinase is activated by uncharged tRNA to phosphorylate the initiation factor eIF-2α, leading to increased translation of Gcn4, a transcriptional activator of general amino acid control. The catalytic subunit of the phosphatase calcineurin is stabilized by its interaction with Hsp90, but overexpressed Hsp90 interferes with its activity and reduced the tolerance of *S. cerevisiae* to salt stress, suggesting a need for fine-tuning the ratio between Hsp90 and its substrate. It is through its interaction with calcineurin, in both *S. cerevisiae* and *Candida albicans*, that Hsp90 facilitates the development of fungal resistance to the azole antimicrobials that inhibit ergosterol biosynthesis (Cowen et al., 2006). The Sba1/p23 cochaperone of Hsp90, which binds to its ATPase domain, protects Hsp90 from its inhibitors geldanamycin and radicicol (Forafonov et al., 2008).

Inducible transcriptional activators constitute a class of substrate proteins that are stabilized and regulated by interaction with Hsp90. One of these is *S. cerevisiae* Mal63, the transcriptional activator of maltose-utilizing genes. Hsp90 binds to Mal63 and stabilizes it, and it is required for

normal growth on limiting maltose medium (Bali et al., 2003). Hap1 is a transcriptional activator that mediates the effects of oxygen on gene expression, and activation of Hap1 by heme depends on its interaction with Hsp90. Among the structural components whose assembly requires Hsp90 are the *S. cerevisiae* core kinetochore complex CBF3 (Lingelbach and Kaplan, 2004) and the actomyosin ring of *S. pombe* (Mishra et al., 2005).

The regulatory interactions of Hsp90 with kinases and transcription factors affect the cellular stress response itself. A two-hybrid screen of the *S. cerevisiae* proteome (Millson et al., 2005) showed that Hsp90 interacted with two stress MAPKs, Hog1 and Slt2. Further analysis showed that binding of Hsp90 to Slt2 increased in heat-shocked cells and proved to be necessary for Slt2 to activate cell integrity-specific gene transcription. Hsp90 was found not to be required for stability and activity of Sty1, the Hog1 ortholog of *S. pombe* (Tatebe and Shiozaki, 2003).

Hsp60

The chaperonin Hsp60 (*E. coli* GroEL) is an essential protein, localized in mitochondria, that is required for the folding of a subset of imported proteins and the assembly of multisubunit enzymes, such as cytochrome *c* reductase and ATPase-ATP synthase (Plesofsky, 2004). During the denaturing conditions of heat shock, Hsp60 helps maintain proteins in their native conformation. In these functions, Hsp60 is aided by Hsp10, which is essential for *S. cerevisiae* growth at 23°C and higher temperatures. Hsp60 forms a tetradecamer, organized into two stacked rings of seven identical subunits each. Binding of unfolded proteins to the hydrophobic apical domains of either ring, in concert with binding of ATP and Hsp10, distorts the structure so that bound peptides move to the hydrophilic interior, where they are released and allowed to refold, accompanied by ATP hydrolysis and Hsp10 dissociation. In addition, Hsp60, which binds to single-stranded DNA at origin of replication sequences, is required for yeast transmission of mitochondrial DNA to daughter cells (Kaufman et al., 2003).

sHsps

The lower-molecular-weight hsps, small hsps (sHsps), are more variable in sequence than other hsp classes, but they have a conserved region, present also in eye lens α-crystallin, that extends from the center almost to the C terminus of the proteins (Plesofsky, 2004). In *N. crassa* and other fungi, there is a nonconserved sequence inserted into this conserved region. Interaction between the two parts of the conserved sequence likely drives the monomeric structure, while the C-terminal portion is responsible for subunit dimerization. The dimers of Hsp26 of *S. cerevisiae* assemble into a 24-subunit sphere-like structure that is activated by high temperature to bind unfolded substrate proteins. High temperature also causes dissociation of Hsp26 into dimers, but this dissociation is not required for chaperone activity (Franzmann et al., 2005). The variable N-terminal region in yeast Hsp26 and *N. crassa* HSP30 is involved in substrate binding. A region in Hsp26 that separates the N terminus from the conserved α-crystallin-related region has been called a thermosensor, since its conformation is most altered by the activating high temperature (Franzmann et al., 2008).

These sHsps differ from the other major chaperone hsps in several ways. Unlike the other hsps, they do not bind and hydrolyze ATP. They have been considered, therefore, to be the first line of defense against the aggregation of heat-denatured proteins, binding substrate proteins but requiring the subsequent cooperation of ATP-dependent chaperones, such as Hsp70, for refolding them. The major sHsps of *S. cerevisiae* and *N. crassa* are not made constitutively, and they are not required for viability, but they are strongly induced by heat shock. The *Aspergillus nidulans* HSP30, however, is expressed constitutively and is only slightly increased by high temperature.

There are two α-crystallin-related sHsps in *S. cerevisiae*, Hsp26 and Hsp42, the latter of which is extended at its N terminus. Like Hsp26, Hsp42 is not made constitutively until the diauxic shift, but it exhibits chaperone activity at both normal and heat shock temperatures, whereas Hsp26 requires high temperature for its activity (Haslbeck et al., 2004). In addition, Hsp42 does not dissociate into dimers at high temperature. Both sHsps are cytosolic under respiratory conditions but nuclear during fermentative growth. Hsp12 of *S. cerevisiae* is not usually grouped with sHsps, because it lacks the requisite α-crystallin domain. However, it has limited homology to sHsps outside the conserved region, and it is induced by the same stimuli. Hsp12 is located in the plasma membrane and protects it from desiccation.

S. cerevisiae Hsp26 has diverged from other sequenced fungal sHsps, showing at most 46 and 41% identity with sHsps of *Kluyveromyces lactis* and *Ashbya gossypii*, respectively. In contrast, *Neurospora* HSP30 showed 54% identity with an sHsp of *Chaetomium globosum* and 51% identity with an sHsp of *Podospora anserina*. Most organisms produce more than one sHsp. In addition to Hsp12, the *A. nidulans* genome encodes five sHsps, three of which are similar to one another (>60% identity) and one of which diverges strongly (Fig. 1). The genome of *N. crassa* encodes three sHsps, which show <40% identity to one another (Fig. 1).

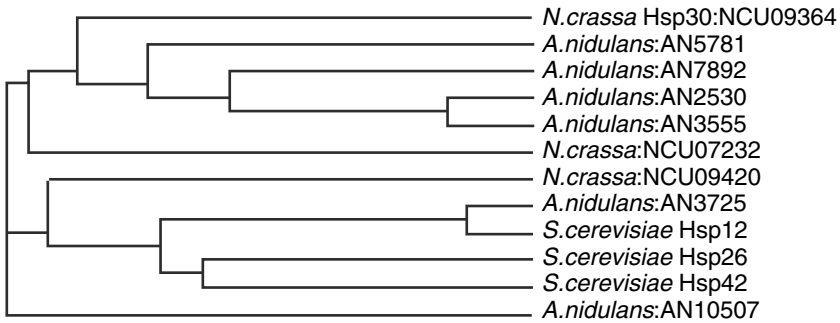

FIGURE 1 A phylogenetic tree generated by ClustalW2 (Larkin et al., 2007) based on multiple sequence alignment of the sHsps of *N. crassa*, *A. nidulans*, and *S. cerevisiae*.

One of the *N. crassa* sHsps (NCU07232), whose expression is heat shock induced, and the divergent *A. nidulans* sHsp (AN10507) are predicted to be mitochondrially localized according to software tools iPSORT (Bannai et al., 2002) and Predotar (Small et al., 2004). Very few fungi encode sHsps similar in size to Hsp42. Homologs of yeast Hsp12, on the other hand, are very common among fungi, including *A. nidulans* and several pathogenic fungi. Its ortholog in *S. pombe*, Hsp9, is induced by nucleotide depletion and DNA damage, in addition to high temperature. The genome of *N. crassa* does not appear to encode an Hsp12 homolog.

No fungal sHsp has been shown to be required for resistance to high temperature, although constitutive expression of Hsp26 slightly increased survival of *S. cerevisiae* at the lethal temperature of 50°C (Plesofsky, 2004). However, production of a truncated HSP30 in *N. crassa*, by the repeat-induced point mutation procedure, caused cells to become extremely sensitive to the dual stress of high temperature coupled with carbohydrate limitation. Compared with the wild type, this mutant strain also showed reduced hexokinase activity at high temperature and reduced import of proteins into mitochondria. Morphological changes affecting the cell walls or membranes of *S. cerevisiae* were observed in *hsp26* and *hsp42* deletion strains (Haslbeck et al., 2004).

Hsp104

Hsp104 (*E. coli* ClpB) is an ATP-dependent chaperone that assembles in the cytosol into a hexamer. It has the unique ability among cellular chaperones to resolubilize protein aggregates after they have formed, rather than simply preventing their formation (Plesofsky, 2004). One of its two nucleotide-binding domains is chiefly responsible for the protein's ATPase activity, while the other domain regulates oligomerization. Hsp104 interacts with the TPR-containing cochaperones of Hsp90 via its C-terminal tail, which is similar to that of Hsp90. Not normally expressed in fermentatively growing yeast cells, Hsp104 is induced by high temperature, but it is made constitutively, as well as being heat shock induced, in respiring cells. An Hsp104-related protein, Hsp78, is located in mitochondria; it displays the same expression pattern as Hsp104. In cooperation with the mitochondrial Hsp70 chaperone complex, Hsp78 is required for protein refolding and for reactivation of the mitochondrial DNA polymerase following cellular heat shock. It is also required for the proteolysis of nonnative peptides of the mitochondrial matrix, which is carried out by the Pim1 protease (Röttgers et al., 2002). Hsp104 and Hsp78 homologs are encoded by *N. crassa* (Table 1) and other fungi.

Ubiquitin

An important aspect of the cellular response to heat shock is the degradation of nonnative proteins. In the cytoplasm this is facilitated by Ssa Hsp70, which helps deliver denatured proteins to the proteasome for degradation (Park et al., 2007). The ligation of ubiquitin, a 76-residue peptide, commonly marks proteins for degradation. Heat shock increases expression of *UBI4*, a polyubiquitin gene that encodes five ubiquitin repeats and is required for *S. cerevisiae* resistance to high temperature and other stresses (Plesofsky, 2004). The four-repeat polyubiquitin gene of *A. nidulans* also is induced by high temperature, but the *N. crassa* polyubiquitin gene was found to be uninduced by heat or other stresses. Expression of two ubiquitin-conjugating enzymes also increases in *S. cerevisiae* in response to heat shock, and at least one of them is required for viability at high temperature. These E2 conjugating enzymes, Ubc4 and -5, are responsible, specifically, for the proteolysis of short-lived and abnormal proteins. The importance of ubiquitin-dependent proteolysis to the heat shock response is shown by the restorative effect of overexpressing *UBI4* on survival of cells that are deficient in hsp synthesis (Friant et al., 2003).

HEAT SHOCK-INDUCED METABOLITES

Trehalose

Trehalose is a nonreducing disaccharide that is a source of stored carbohydrate in fungal spores. It also accumulates in fungal cells, such as those of *S. cerevisiae*, *N. crassa*, *A. nidulans*, and *Botrytis cinerea* in response to high temperature and other stresses; this accumulation in *N. crassa* is accompanied by glycogen degradation (Plesofsky, 2004). Trehalose protects cells against thermal and oxidative stresses and increases spore viability. Trehalose accumulation has been shown to protect proteins from oxidative damage and to suppress aggregation of denatured proteins, facilitating their chaperone-mediated refolding. The rapid cellular degradation of trehalose upon return to normal temperature is advantageous, however, since continued high trehalose levels reduce thermotolerance by interfering with subsequent refolding of proteins. Infectivity of the human pathogens *Cryptococcus neoformans* (Petzold et al., 2006) and *C. albicans* (Zaragosa et al., 1998) is strongly decreased by disruption of trehalose biosynthesis, which is required for growth at 37°C. Deletion of the *TPS2* gene of *S. cerevisiae* was found to be particularly deleterious under the inducing heat stress, due to accumulation of the toxic intermediate, trehalose-6-phosphate. The regulation of stress-induced trehalose synthesis differs among fungi. Both subunits of the trehalose-6-phosphate synthase/phosphatase complex are induced by heat shock in *S. cerevisiae* and *C. neoformans* (Petzold et al., 2006), whereas only one (subunit B) is induced in *Aspergillus niger* and the main synthase subunit (subunit A) in *A. nidulans* shows increased activity, but not expression.

One difficulty in assessing the thermoprotective role of trehalose has been that strains defective in trehalose synthesis are also impaired in induction of heat shock genes. An explanation for this linkage is that trehalose apparently functions as a positive regulator of the yeast heat shock transcription factor, Hsf1, at high temperature (Conlin and Nelson, 2007). Increased levels of trehalose (up to a point) generate increased levels of transcripts for hsps. This is accompanied by increased phosphorylation of Hsf1, possibly due to its conformation being altered by trehalose. The alternative stress-activated transcription factors, Msn2 and Msn4, are not affected by trehalose.

Sphingolipids

That sphingolipids may have a role in the survival of fungi during stress was suggested by the inability of yeast, deficient in sphingolipid biosynthesis, to grow at high temperature, despite suppressor mutations allowing growth at normal temperature. Unlike wild-type *S. cerevisiae*, which accumulated sphingoid bases and ceramides upon heat stress, the sphingolipid-deficient suppressor mutant, *lcb1-100*, did not arrest in G_0/G_1 in response to heat shock (Jenkins and Hannun, 2001). Furthermore, the synthesis of hsps by *lcb1-100* was reduced at high temperature, due to a defect in translation initiation (Meier et al., 2006). Both the cell cycle arrest and translation defects were due to a lack of sphingoid bases, and it is likely that the translation defect is due to reduced activity of the sphingoid-base-activated Pkh kinases and their downstream Ypk target.

No increase in sphingolipids was evident in *N. crassa* when heat stressed in minimal medium (Plesofsky et al., 2008). However, sphingolipid synthesis by *S. cerevisiae* at high temperature was found to be influenced by the nature of the growth medium, particularly the inclusion of serine, a substrate of sphingolipid synthesis (Cowart and Hannun, 2007). Although heat shock of *N. crassa* seemed to decrease some classes of ceramide, the combination of heat shock with carbohydrate deficiency, as a dual stress, induced the synthesis of a unique C_{18}-phytoceramide by cells that subsequently did not recover from the stresses. There is evidence that this phytoceramide may be involved in cell death signaling (Plesofsky et al., 2008).

REGULATION

Heat Shock Transcription Factor

The transcription of genes encoding hsps is governed by the heat shock transcription factor (HSF). Other classes of genes whose heat-induced transcription in yeast is regulated by Hsf1 encode proteins involved in proteolysis, energy generation, carbohydrate metabolism, and cell wall integrity (Yamamoto et al., 2005). Oxidative stress (Yamamoto et al., 2007) and glucose starvation (Hahn and Thiele, 2004) also activate HSF. HSF binds as a trimer to heat shock elements (HSEs) within target gene promoters. The fundamental unit of the HSE is nGAAn repeated in tandem on alternating DNA strands (perfect HSE), with a minimum of three pentanucleotides being required in *S. cerevisiae* for activity and a five-nucleotide gap between two of the pentanucleotides still supporting induction (gapped HSE). However, the promoter regions of genes that depend on yeast Hsf1 for transcription were found to have other variations from these canonical HSEs. These include three pentanucleotides with five nucleotide gaps between each (stepped HSE) (Yamamoto et al., 2005).

The monomeric structure of Hsf1 includes a winged-helix-turn-helix DNA-binding domain and independent N- and C-terminal activation domains, which display different specificities for hsp gene transcription (Eastmond and Nelson, 2006). Deletion of the C-terminal domain of Hsf1 restricted growth of *S. cerevisiae* to 35°C and below, primarily due to loss of Hsp90 synthesis. There are, as well, a repressor domain (CE2) and a C-terminal domain that alleviates that repression (CTM). Oligomerization into DNA-binding trimers is through a leucine zipper-like hydrophobic repeat that forms a coiled coil during oligomerization (Plesofsky, 2004). Whereas oligomerization is constitutive in yeast Hsf1, the HSFs of other eukaryotes include a C-terminal leucine zipper that interferes with oligomerization through intramolecular coiled-coil formation, until the HSF is activated. Since Hsp70 and Hsp90 repress HSF, it becomes activated by high temperature and other protein-denaturing stresses that divert hsps to function as chaperones. Activation of HSF is accompanied by its increased phosphorylation.

There is only one HSF gene in the yeasts *S. cerevisiae* and *S. pombe*, and it is essential for cell survival, being required for the basal expression of essential hsps, such as Hsp70 and Hsp90. There are two HSF-like genes in *N. crassa* (Borkovich et al., 2004). One (NCU08512) encodes HSF1, which is moderately conserved and appears to have three regions with coiled-coil propensity (COILS), suggesting that it requires activation for homotrimer formation, unlike the *S. cerevisiae* HSF. The second (NCU08480) encodes HSF2, which has an HSF-like DNA-binding domain and two regions predicted to form coiled coils. Only HSF1 was found to be essential for *N. crassa* survival, but HSF2 is required for conidial development (Thompson et al., 2008). Fungi also encode a protein that contains the HSF DNA-binding domain coupled with a two-component response regulator receiver domain. This protein in *S. cerevisiae* and in *N. crassa*, Skn7 and RRG-2, respectively, enhances resistance to oxidative stress (Raitt et al., 2000; Banno et al., 2007).

Although yeast Hsf1 binds constitutively to promoters of heat shock genes, especially highly expressed ones, its binding strongly increases in response to stress. There is also dramatic chromatin remodeling, coincident with Hsf1 binding, at the promoters of heat shock genes; this involves decreased nucleosome occupancy and moderately increased acetylation of histone H4 (Shivaswamy and Iyer, 2008). The promoters of genes repressed by heat shock, most notably ribosomal protein genes, showed the opposite chromatin effects. These changes in chromatin structure were found to be mediated by the yeast SWI/SNF complex.

General Stress Response

Many of the yeast genes whose expression is mediated by Hsf1 are also regulated by Msn2 and Msn4, zinc finger-containing transcriptional activators, in the general stress response. These transcription factors become activated and hyperphosphorylated during stationary phase and by a variety of stress stimuli that overlap with Hsf1-inducers. Msn2 and Msn4, which are partially redundant, bind to the promoter stress response element (STRE), CCCCT (Plesofsky, 2004). Deletion of *MSN2* and *MSN4* was found to affect expression of Hsp12 more than other hsps (Erkina et al., 2008). High levels of cyclic AMP that activate PKA are negative regulators of Msn2/4, and mutant yeast cells that lack cyclic AMP display increased thermotolerance and an increased life span that depend on Msn2/4. PKA was also found to repress, under nonstress conditions, the Hsf1-dependent expression of Hsp12 and Hsp26, but not other hsps (Ferguson et al., 2005).

The regulatory characteristics of this general stress response (Msn2/4) and the specific heat shock response (Hsf1) display notable differences. Protein misfolding, caused by amino acid analogs, led to Hsf1-mediated gene induction but not to Msn2/4 induction. Furthermore, repression of ribosomal protein genes during stationary phase is characterized by increased histone H4 acetylation at promoters, whereas repression during heat shock involves decreased H4 acetylation. The SWI/SNF complex is not recruited to promoters of genes during their induction or repression by stationary phase (Shivaswamy and Iyer, 2008).

The general stress response of yeast and the multiple stimuli that activate Hsf1 contribute to the cross-protection against other stresses displayed by *S. cerevisiae*, upon exposure to a single type of stress. There is no evidence, however, that other fungi utilize Msn-like transcription factors or STRE elements for general stress gene expression. *Trichoderma atroviride* encodes a protein, Seb1, whose zinc finger domain is like that of Msn2/4. Its expression increases during osmotic stress, and it enhances this stress resistance. Although Seb1 binds to the AGGGG element present in chitinase gene promoters, it does not affect gene transcription (Peterbauer et al., 2002). *C. albicans* also encodes proteins with Msn-like zinc finger domains, and several stress-related genes contain STRE elements, but these proteins do not play a role in the transcriptional response to heat or other stresses (Nicholls et al., 2004). In *Candida glabrata*, on the other hand, Msn2 and -4 contribute to oxidative stress resistance (Cuéllar-Cruz et al., 2008). Proteins were identified in *N. crassa* that bind to a STRE element in

the promoter of the glycogen synthase gene, which is repressed by heat shock (Freitas et al., 2008). Interestingly, one of these proteins is a J protein (NCU02424) without an obvious counterpart in *S. cerevisiae*.

Stress MAPKs

MAPKs and their activating MAPKKs and MAPKKKs constitute a shared mechanism by which fungi respond to and signal stress. *S. cerevisiae* and other fungi utilize two stress MAPK signaling systems (Plesofsky, 2004). Hog1, in the high-osmolarity glycerol (HOG) pathway, responds to hypertonic stress, and its effects are mediated in concert with the Msn2/4 general stress response. Slt2 is in the cell wall integrity pathway, and it responds to hypotonic stress and other perturbations of the cell surface; this pathway is dependent on PKC1. Both these MAPKs respond to heat stress, but in *S. cerevisiae* Hog1 shows a weaker response, with its signaling of osmotic stress being primary.

Although the *S. cerevisiae* Hog1 is activated primarily by hyperosmotic stress, it is also activated by oxidative, citric acid, and heat stress. When activated, phosphorylated Hog1 translocates into the nucleus, where it is recruited to specific promoters and regulates transcription factors and gene expression. However, it does not translocate into the nucleus when high temperature is the inducing stress (Winkler et al., 2002). Unlike Hog1, Sty1 (Hog1 ortholog) of *S. pombe* is strongly activated by a wide variety of stresses, including hyperosmolarity, heat stress, UV, oxidative stress, and starvation (Sansó et al., 2008). Sty1 associates with promoters of induced nuclear genes, whether it is activated by heat shock or by other stresses. Hog1 orthologs of *C. neoformans* and *N. crassa* (OS-2) also respond to diverse stresses, including heat stress (Bahn et al., 2007; Noguchi et al., 2007), and that of *N. crassa* is further activated by glucose deprivation (Plesofsky et al., 2008). The Hog1 of *C. albicans* is activated by oxidative, osmotic, and heavy metal stress, but it is not activated by high temperature (Enjalbert et al., 2006).

S. cerevisiae Hog1 is regulated by the MAPKK Pbs2 and by three functionally overlapping MAPKKKs, Ssk2/Ssk22 and Ste11 (Fig. 2). Two membrane-spanning proteins, Sho1 and Sln1, independently respond to stress and converge to activate Pbs2 via different MAPKKKs. These two upstream regulatory branches, Sho1-Ste11 and Sln1-Ssk2/Ssk22, of *S. cerevisiae* are redundant for conferring resistance to hyperosmolarity, but they differ in their signaling of other stresses (O'Rourke and Herskowitz, 2004). Sho1 is an SH3-containing protein, and Sln1 is a histidine kinase; it is Sho1 that is responsible for activating Hog1 in response to heat stress (Winkler et al., 2002). OS-1 of *N. crassa* is a histidine kinase, like Sln1, that is required for resistance to osmotic stress and for hyperosmolarity-induced phosphorylation of OS-2 via OS-4 and -5 (Fig. 2). However, OS-1 (like yeast Sln1) is not required for activating the MAPK cascade and OS-2 in response to heat shock (Noguchi et al., 2007). The activation of OS-2 by heat shock leads to increased expression of the same genes that are induced by high osmolarity.

Sty1 of *S. pombe* is regulated by three MAPKKKs, which all activate the MAPKK Wis1 (Sansó et al., 2008). Hog1 orthologs of other fungi, such as *N. crassa*, *A. nidulans*, *C. albicans*, and *C. neoformans*, in contrast, are regulated by only one MAPKKK Ssk2, which activates the MAPKK Pbs2 (Noguchi et al., 2007; Furukawa et al., 2005; Cheetham et al., 2007; Bahn et al., 2007). Inactivation of Hog1 and Sty1 (Fig. 2) is regulated by tyrosine phosphatases (*S. cerevisiae* Ptp2 and -3 and *S. pombe* Pyp1) and 2C serine/threonine phosphatases (*S. cerevisiae* Ptc1, -2, and -3 and *S. pombe* Ptc1) (Plesofsky, 2004). Inhibition of the Pyp1 tyrosine phosphatase appears to be the chief means of activating Sty1 in response to heat shock. However, unregulated activation of Hog1, due to deletion of the tyrosine phosphatase genes *PTP2* and *PTP3*, made heat shock lethal to *S. cerevisiae*.

In addition to their sensitivity to hyperosmotic stress, cells with *HOG1* deletion recovered from heat stress more slowly than the wild type (Winkler et al., 2002). *S. pombe* cells with *STY1* deletion became extremely sensitive to heat, oxidative, and osmotic stresses; and some of this sensitivity appears to be mediated by the Atf1 transcription factor, which is a major substrate of Sty1 (Sansó et al., 2008). Mutation of OS-2 or its activating kinases, OS-4 (Ssk2) and OS-5 (Pbs2), makes *N. crassa* sensitive to osmotic stress (Noguchi et al., 2007). These mutant strains are resistant, however, to phenylpyrrole fungicides, and *os-2* mutants survive combined heat and nutrient stresses better than wild-type cells (Plesofsky et al., 2008). *C. neoformans* is unusual in having strains that constitutively phosphorylate Hog1 due to an active Ssk2.

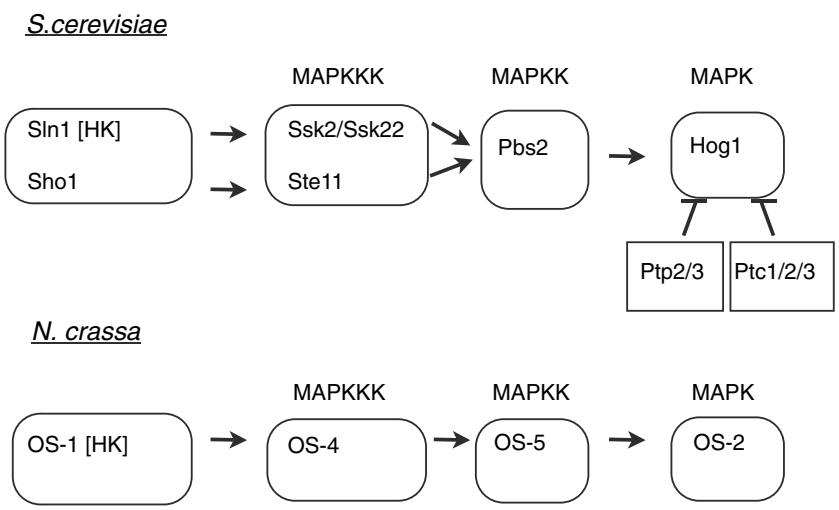

FIGURE 2 The major stress MAPK pathways of *S. cerevisiae* and *N. crassa*. HK, histidine kinase.

This constitutive phosphorylation made the *C. neoformans* strains particularly resistant to high temperature and other stresses (Bahn et al., 2007). Deletion of *A. nidulans sakA* (Hog1 ortholog), which is activated by both osmotic and oxidative stresses, did not make cells sensitive to hyperosmotic stress. However, the mutant conidia, which lost viability during storage, were sensitive to oxidative and heat stress.

The cell integrity MAPK pathway of *S. cerevisiae*, consisting of one MAPKKK, two redundant MAPKKs, and the Slt2 MAPK, is activated in response to many stresses, such as high temperature and hypo-osmolarity, that perturb the cell surface. PKC is typically responsible for activating this pathway; and the activated Slt2 induces transcription of genes, through Rlm1 and Swi4, that direct cell wall remodeling and cell cycle arrest (Harrison et al., 2004). However, activation of Slt2 by heat shock does not require either PKC or MAPKKK (Harrison et al., 2004). Slt2 activation during yeast bud formation is regulated by Ptc1, a serine/threonine phosphatase that also regulates Hog1 (Du et al., 2006). The tyrosine phosphatases Ptp2 and -3 also negatively regulate both Slt2 and Hog1. This shared inactivation mechanism prevents inappropriate activation of one MAPK by another MAPK pathway (Winkler et al., 2002). In addition, there are two dual-specificity (tyrosine and threonine) phosphatases, Sdp1 and Msg5, that contribute to inactivating Slt2. Sdp1, whose stress induction depends on Msn2/4, dephosphorylates heat-activated Slt2 (Hahn and Thiele, 2002). Msg5, which becomes phosphorylated by activated Slt2, maintains the low basal level of uninduced Slt2 activity (Flandez et al., 2004).

Other types of communication between these two stress MAPK pathways have been described for *S. cerevisiae* and *S. pombe*. Hog1 is required for the transcriptional induction of *SLT2* by hyperosmolarity, but not by heat stress (Hahn and Thiele, 2002). In contrast, the *S. pombe* Slt2 homolog, Spm1, which responds to a wide variety of stresses, requires Sty1 (through Atf1) for inactivation after hyperosmotic stress (Madrid et al., 2006). In another example of regulatory communication, the activation of Slt2, which is needed for cell growth at high temperature, requires both the phosphorylation of Slt2 and the Hsf1-directed synthesis of Hsp90, since Hsp90 is an activating chaperone for phosphorylated Slt2 (Truman et al., 2007).

CONCLUSIONS

Our growing understanding of how organisms respond to the stresses they encounter in their natural environment gives us an enhanced appreciation of their flexibility and adaptability outside the optimal conditions of the laboratory. Heat shock was the first stimulus that was serendipitously observed to elicit this adaptive response in gene expression, followed by investigation of many other stresses, including those caused by reactive oxygen species, high and low osmolarity, cold, and heavy metals. *S. cerevisiae* has proved to be a very useful organism for understanding the functions of individual hsps, how they are protective against the inducing stress and other stresses, and what their roles are during cell growth and development. The complex mechanisms employed to regulate expression of stress-induced genes are also being clarified by their analysis in *S. cerevisiae*.

Constitutively made hsps will continue to have new functions discovered in the course of analyzing normal (nonstress) fungal growth, development, reproduction, and death. New cochaperones for particular activities will also be identified. Although genomic sequencing and studies in other fungi indicate that the major hsps and regulatory mechanisms for their expression are generally conserved, there are some significant exceptions. Especially fertile ground for scientific discovery should come from deeper and broader understanding of how responses to heat and other stresses are regulated in fungi other than *S. cerevisiae*. It is in these regulatory components that differences among organisms should be expected, in part because they occupy different environments. Where particular extremes are a common part of an organism's environment, greater safeguards should already exist independently of a stress response. This applies, for example, to hyperosmotic stress for fungi that are storage molds and to high-temperature stress for human pathogens. The response of human and plant pathogens to oxidative stress appears to be especially important for resistance to defensive host mechanisms.

There is extensive overlap between the gene expression profiles and signaling pathways induced by heat, oxidative, osmotic, and other stresses. This is partly because one initial stress can lead to other stresses. For example, both high-temperature stress and glucose starvation result in increased reactive oxygen species and oxidative stress. In addition, there are basic cellular components, proteins and membrane lipids, that are vulnerable to several stresses. It is not surprising, therefore, that organisms utilize similar strategies for different stresses, although the balance among the various pathways and molecules is fine-tuned for a particular stress.

Although the stress response is adaptive and protective in the short term, it is detrimental in the long term, involving an inhibition of normal protein synthesis and interruption of the cell cycle while still expending energy. Careful controls are built into the response, so that it is transient, continuing as long as it is needed, but allowing a return to normal cellular activities. If these responses were unregulated, they would be detrimental, rather than helpful, as seen when there is a buildup of trehalose, particular sphingolipids, Hsp90, or a hyperactivated Hog1 MAPK. Another potential limitation to the stress response is that it may become overwhelmed by concurrent exposure to multiple stresses. Exploring these limits to what the heat shock response can accomplish will expand the range of what we can learn about how stress responses aid survival.

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SEXUAL DEVELOPMENT

VIII

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Mating Systems and Sexual Morphogenesis in Ascomycetes

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Ascomycetes, along with Basidiomycetes, most probably diversified when exiting water at about the same time as photosynthetic organisms, be they the ancestors of present-day alga-forming lichens (Lutzoni et al., 2001) or land plants (Taylor and Berbee, 2006). Whether Ascomycetes now live as saprobes on dead organic material or as parasites or mutualists of other organisms, they have had to solve the problem of dispersal in air in their new terrestrial biotope. To do so, their ancestors lost the flagella that permitted their dispersal in water and have evolved effective asexual and sexual dispersal mechanisms, through the production of either mitospores or meiospores. The production of multicellular sexual fruiting bodies ensuring effective dispersal in air is a hallmark of these "superior" fungi, but many species have lost the ability to build complex reproductive structures. Sexual development requires that the mycelium continuously function as an information network. Initiation of the formation of sexual fructifications usually requires the sensing of medium exhaustion. Fertilization of the initial sexual structures occurs after finding a sexually compatible partner, which is often a different individual for self-incompatible species. Completion of the building of fructifications requires cross talk between the mycelium, the envelope of the fructification, and the meiotic tissues. The mycelium provides nutrients to the envelope and meiotic tissues, while these tissues are likely to forward continuous cues to the envelope and to the mycelium to get a continuous supply of nutrients. Meiotic tissues undergo surprisingly complex cellular transitions, some of them under the control of mating-type genes, to ensure the formation of some hundreds of progeny from a single fertilization event.

The intention here is to give readers an up-to-date review of these steps in Pezizomycotina, from the differentiation of reproductive cells to the development of the fructification. However, the bulk of the review deals with mating-type structures and their function in self-incompatible, self-compatible, and supposedly asexual fungi, as these three classes of fungi have been the targets of many studies and mating-type genes are key elements acting throughout the entire process, from partner selection to the proper development of fructifications. The *Cochliobolus heterostrophus* and *Podospora anserina* mating systems are not discussed extensively, as they have been recently reviewed in depth in *Sex in Fungi* (Turgeon and Debuchy, 2007). The most important conclusions, however, are presented here and are referred to when relevant in the discussion of other mating systems. The evolution of mating types was reviewed in two chapters in *Sex in Fungi* (Butler, 2007; Turgeon and Debuchy, 2007), and recent progress in this field has been commented upon recently (Casselton, 2008; Dyer, 2008). Therefore, this topic is not extensively reviewed in this chapter. Previous reviews on mating types are listed in Debuchy and Turgeon (2006). The reader is also directed to the seminal reviews by Robert L. Metzenberg and N. Louise Glass on mating types (Metzenberg and Glass, 1990) and by Paul Dyer et al. on sexual morphogenesis (Dyer et al., 1992) for further reading.

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INITIATION OF SEXUAL REPRODUCTION

Overview of Male and Female Gamete Formation

Very little is known about the differentiation of gametes and gametangia in ascomycetes. Cytological studies have uncovered several kinds of male and female reproductive structures. The female gametangium, the ascogonium, is usually a large multinucleate cell with an elongated multicellular extension, the trichogyne. The trichogenous hypha grows towards the

male cell, coils around it, and collects its nucleus, which migrates towards the body of the ascogonium. The trichogyne exhibits few, if any, differences with vegetative hyphae and is mostly characterized by its capacity to grow when the vegetative hyphae are in stationary phase. In some species, true trichogynes are lacking, but the upper cells of the ascogonium produce multicellular hyphae, the copulating hyphae, that are branched and may attain a length of 2 mm (Gaümann, 1952). The copulation hyphae may be distinguished from the vegetative hyphae by their smaller diameter and more fragile appearance. In many instances, unfertilized ascogonia recruit neighboring hyphae to form small hyphal structures in which the ascogonium is embedded and in which the protected gametangium awaits fertilization (the protoperithecium or the protoapothecium). The male cell can be a specialized hypha (antheridium), an asexual vegetative spore that may germinate (microconidium or macroconidium) or not (spermatium) (in the latter case it is dedicated solely to sexual reproduction), or an ascospore (Gaümann, 1952; Lee et al., 2008). When sexual or asexual cells are absent or nonfunctional, broken mycelium has been successfully used as the nucleus donor (Bouhouche et al., 2004), indicating that even in species differentiating male gametes, fertilization may be brought about by any vegetative hyphae. Usually, thalli are hermaphroditic, differentiating both male and female gametangia, and due to the size difference between the male and female reproductive structures, the reproduction style in filamentous ascomycetes is considered to be anisogamous.

Male and female gametes are not constitutively formed on the vegetative filaments, and the starting point of their differentiation relies on a plethora of variables, among which external factors are of utmost importance (Klebs, 1898). A brief overview of these factors is given in the next sections. The reproductive structures may be induced when the mycelium comes into close contact with a compatible partner. In *Ascobolus stercorarius*, the formation of antheridia and the differentiation of ascogonia from vegetative hyphae are induced by placing a strain near a compatible partner (Bistis and Raper, 1963). The self-incompatible *Nectria haematococca* var. *cucurbitae* does not rely on contact with its partner for differentiating protoperithecia, but its oidia undergo a change in morphology when placed on a compatible mycelium, appearing to acquire some features of sexual cells (Bistis, 1979). Surprisingly, the requirement for a contact between two cells has also been described in the self-compatible *Sordaria brefeldii*. In this species, ascogonia are present but functionless and they no longer initiate the development of the fructification. Instead, perithecia are formed upon mating between two hyphae at any place in the mycelium (Dengler, 1937). Taken together, these data suggest that the initiation of reproductive structure formation results from a complex cross talk between the cells and their environment.

Male Gamete Formation

External Factors Triggering Male Gamete Formation

Data on how various external factors affect male gamete differentiation are sparse and are mostly due to fortuitous observations (Drayton, 1932; Esser, 1974). External factors triggering microconidiation and macroconidiation have been investigated more thoroughly, with most studies centering on *Neurospora crassa*. The isolation of *N. crassa* mutants that selectively produce microconidia has greatly aided microconidiation studies (reviewed by Maheshwari, 1999). Humidity, temperature, chemicals, and starvation favor *N. crassa* microconidiation (see Maheshwari, 1999, and references therein). In fact, a shift from a high to a lower temperature, addition of iodoacetate in the media, and low carbon and ammonium nitrogen concentrations are optimum conditions for *N. crassa* microconidiation (Rossier et al., 1977; Ebbole and Sachs, 1990; Maheshwari, 1999). Conversely, microconidiation is inhibited on rich medium.

Genetic Control of Male Gamete Formation

The genetic basis of male gamete differentiation has been explored in *P. anserina* and *N. crassa*. *P. anserina* is a producer of spermatia, while *N. crassa* produces micro- and macroconidia but no spermatia (reviewed by Maheshwari, 1999). Surprisingly, deletions of the mating-type locus in the self-incompatible *P. anserina* (Coppin et al., 1993) and *N. crassa* (Ferreira et al., 1998) have shown that mating-type genes are not necessary for male gamete differentiation. Spermatia and microconidia develop on special hyphae, which are reminiscent of conidiophores. This suggests that spermatia and conidia may be formed by a common pathway. This idea is supported by the fact that *Ami1* (Graia et al., 2000), a gene required for the formation of functional spermatia in *P. anserina*, is a functional ortholog of *Aps*A. ApsA is involved in nuclear positioning in vegetative hyphae and during conidiogenesis in *Aspergillus nidulans* (Fischer and Timberlake, 1995). *Ami1* mutants are sterile because migration of nuclei in the developing spermatia is abolished. This mutant also has incorrect nuclear positions at other stages of the sexual cycle. This defect in vegetative hyphae is barely detectable, if at all, raising the possibility that the function of Ami1 is restricted to the sexual stage and to the formation of reproductive structures. However, the *A. nidulans aps*A mutant has no sexual defect. In *P. anserina*, *PAH1*, encoding a homeodomain transcription factor, acts as a repressor in spermatium formation (Arnaise et al., 2001b), i.e., a knockout of this gene results in a 10-fold increase in the number of spermatia, and its overexpression leads to the absence of spermatium production. The ortholog of this gene (*kal-1*: NCU03593) has been characterized in *N. crassa* (Colot et al., 2006) and shown to influence macroconidiation and colony pigmentation and morphology. Although the Δ*kal-1* mutant produces macroconidia and is male fertile, it is not yet known whether this mutant exhibits any subtle defects in male fertility or macroconidiation or is affected in microconidia production. Further investigation of the Δ*kal-1* phenotype may reveal a link between spermatia and conidiation pathways.

Female Organ Differentiation

External Factors Triggering Female Gamete and Fruiting-Body Formation

The formation of the female gamete and the correct development of the fruiting body from the fertilized ascogonium necessitate proper conditions during mycelium growth and also up to and including the maturation of the ascospores. Very few studies have focused on the differentiation of ascogonia per se; rather, most studies look at the successful production of fruiting bodies, in terms of number and maturation. The major environmental factors (nutrients, pH, atmospheric conditions, and light) have been investigated.

Nutrient Requirements

Successful reproduction in ascomycetes requires a set of diverse factors, among which, as very nicely illustrated by Lee and Honigberg (Lee and Honigberg, 1996), nutrient availability is crucial. These authors have shown that, once engaged in meiosis (after starvation), *Saccharomyces*

cerevisiae cells can reenter the vegetative growth cycle when shifted back to a rich medium until the meiosis I division stage. In filamentous ascomycetes, the role of nutrients in fructification induction has been studied mainly in Sordariomycetes: *N. crassa* (Hirsch, 1954), *Sordaria fimicola* (Hall, 1971), *Arachniotus albicans* (Lacoste and Dujardin, 1972), *Hypomyces solani* (syn. *Haematonectria haematococca*) (Hix and Baker, 1964), and *Nectria galligena* (Dehorter, 1972) (for a review see Moore-Landecker, 1992). Their role in initiating reproduction has more recently been studied in the eurotiomycete *A. nidulans* (Han et al., 2003). In particular, the effects of the carbon/nitrogen (C/N) ratio on sexual reproductive body differentiation have been studied. The overall findings show that there is an optimum C/N ratio (nearly 10, depending on the ascomycete) to engage in sexual reproduction. However, sexual development initiation is often delayed or impaired on rich medium (high C and N concentrations), even with an optimal C/N ratio. Nevertheless, recent data suggest that high-nutrient conditions favor *A. nidulans* sexual reproduction but nutrient limitations induce asexual reproduction (Han et al., 2003).

Filamentous ascomycetes can usually use various compounds for sources of C and N; nevertheless, some fungi require particular C and N sources for their reproduction (Moore-Landecker, 1992). Beyond the optimum C/N ratio requirement, other compounds, for example, vitamins (biotin and thiamine) (Bretzloff, 1954; Dehorter, 1972; Asina et al., 1977), minerals (iron and zinc), or even fatty acids (Dyer et al., 1993; Goodrich-Tanrikulu et al., 1998; Bowden and Leslie, 1999; Hynes et al., 2008) and caffeine, may also be required to initiate sexual differentiation of some ascomycetes. Interestingly, caffeine simultaneously triggers *Emericella unguis* cleistothecial formation and represses conidial formation (Kakkar and Mehrotra, 1971). There are suggestions for a similar link between external factors and the balance between conidiation and sexual reproduction in *A. nidulans*. In fact, deletion of the *ppoA* gene, encoding a fatty acid dioxygenase involved in the biosynthesis of the linoleic acid-derived psiBα oxylipin, stimulates asexual development. Conversely, *ppoA* overexpression increases meiospore production (Tsitsigiannis et al., 2004a, 2004b). Even if the regulation of the balance between sexual and asexual development in *A. nidulans* is a complex mechanism requiring several genes (Tsitsigiannis et al., 2005), we can imagine that addition of fatty acids to the culture medium may influence cellular fatty acid concentrations; cellular fatty acid concentrations may in turn influence the oxylipin balance and, in the case of linoleic acid addition, trigger sexual reproduction by increasing psiBα.

pH and Atmospheric Conditions

The influence of pH and atmospheric conditions on female organ induction is highly variable and is usually linked to the environmental and temporal cycle of the fungus in nature. Initiation of sexual reproduction in the sordariomycete *A. albicans*, as it is for several ascomycetes, is clearly not dependent on a particular pH range (Lockwood, 1937; Lacoste and Dujardin, 1972; Moore-Landecker, 1992). However, while the pH appears to have limited effect on the number and maturation of *A. albicans* perithecia, there is usually an optimum pH to produce abundant fruit bodies with mature ascospores in most ascomycetes. Comparative studies performed with ascomycete species under various environmental conditions in nature show that the optimum pH required for their sexual reproduction is correlated with the composition (alkaline or not) of their natural habitat.

Production of female organs usually occurs over a narrow temperature range (20 to 24°C) in a high relative humidity (65 to 100%). However, some ascomycetes fruit at higher or lower temperatures (*Chaetomium aureum* can produce cleistothecia at 35°C; *Thelebolus* species can form apothecia at 0°C) and others required a cold exposure to trigger ascocarp production. Changes to atmospheric conditions generate morphogenic responses in various fungi. In particular, variation of CO_2 and O_2 concentrations can either inhibit or induce reproductive structures (Robertson and Emerson, 1982). In *A. nidulans*, the balance between sexual and asexual reproduction is influenced by atmospheric conditions (Champe et al., 1994; Han et al., 2003). Han and colleagues have shown that limiting oxygen during *A. nidulans* growth is responsible for an irreversible commitment to sexual reproduction, and their overall observations suggest that sexual reproduction preferentially occurs during a reduced rate of aerobic respiration (Han et al., 2003).

Light

In many fungi, light is one of the prominent physical factors controlling sexual reproduction, either by stimulating or inhibiting the formation of reproductive structures. The intensity and time of exposure to light are usually of utmost importance in species whose sexual induction is light dependent (see Moore-Landecker, 1992, for a review). Conversely, inhibition of sexual reproduction by light has been clearly demonstrated in other species. Careful observation of *Nannizzia incurvata* sexual development has shown that light prevents the embedding of the female gametangium around the male cell (Kwon-Chung, 1969). Nevertheless, some fungi, such as *P. anserina* or *A. albicans* (Lacoste and Dujardin, 1972), can initiate and complete their whole sexual cycle without light. However, under dark conditions, *P. anserina* exhibits a 48-h delay in the progression of its sexual cycle and produces fruiting bodies that are neck deficient and fewer in number (V. Berteaux-Lecellier, unpublished results). Fructification formation in *Gelasinospora reticulospora* illustrates a more complex situation: continuous white light prevents their formation, and some female organs are differentiated in total darkness; however, their formation is highly enhanced on white-light exposure after a dark period (Inoue and Furuya, 1970). The effect of light (wavelength and time of exposure) on sexual development has been extensively studied in *N. crassa* and *A. nidulans*. Also, various receptors involved in the mechanism of light perception have been studied, including their potential link to G-protein-mediated signaling (Bieszke et al., 1999a, 1999b; Cheng et al., 2003; Blumenstein et al., 2005; Seo and Yu, 2006). WC-1, the circadian blue-light receptor in *N. crassa*, has been shown to influence the abundance of protoperithecia (Degli-Innocenti and Russo, 1984; Ballario et al., 1996; Oda and Hasunuma, 1997). However, the physiological role of most of the *N. crassa* photoreceptors (Borkovich et al., 2004) is still unknown. In *A. nidulans*, sexual fructification formation is stimulated in darkness. Conversely, red light stimulates asexual development and inhibits sexual reproduction. This regulation requires the *fphA* gene, encoding the red- and far-red-light photoreceptor. As expected, *fphA* deletion partially suppresses sexual development repression by red light. However, there are fewer fruiting bodies under red-light conditions than in darkness, suggesting that another red-light-sensing system exists (Mooney and Yager, 1990; Han et al., 2003; Blumenstein et al., 2005). The coordination of sexual development with light is affected by the velvet complex, which may participate in the remodeling of chromatin (Bayram et al., 2008).

Genetic Control of Female Gamete Formation

Differentiation of the ascogonium is even less understood than that of male gametes. Deletion of the mating-type genes in *P. anserina* (Coppin et al., 1993), *N. crassa* (Ferreira et al., 1998), and the self-compatible *Gibberella zeae* (Desjardins et al., 2004) has demonstrated that ascogonium formation does not depend on these genes. In *N. crassa*, the MAK-1 and OS-2 mitogen-activated protein kinases (MAPKs) are required for the proper differentiation of ascogonia (Maerz et al., 2008). Interestingly, the orthologous genes in *P. anserina* are not involved in ascogonium development (Kicka and Silar, 2004; Jamet-Vierny et al., 2007) (P. Silar, unpublished result). Genes involved in protoperithecial development have been identified. These genes are also involved in subsequent steps of the formation of the fructification envelope (see "Making the Envelope" below).

Fruiting-Body Ontogeny, Shape, and Evolution

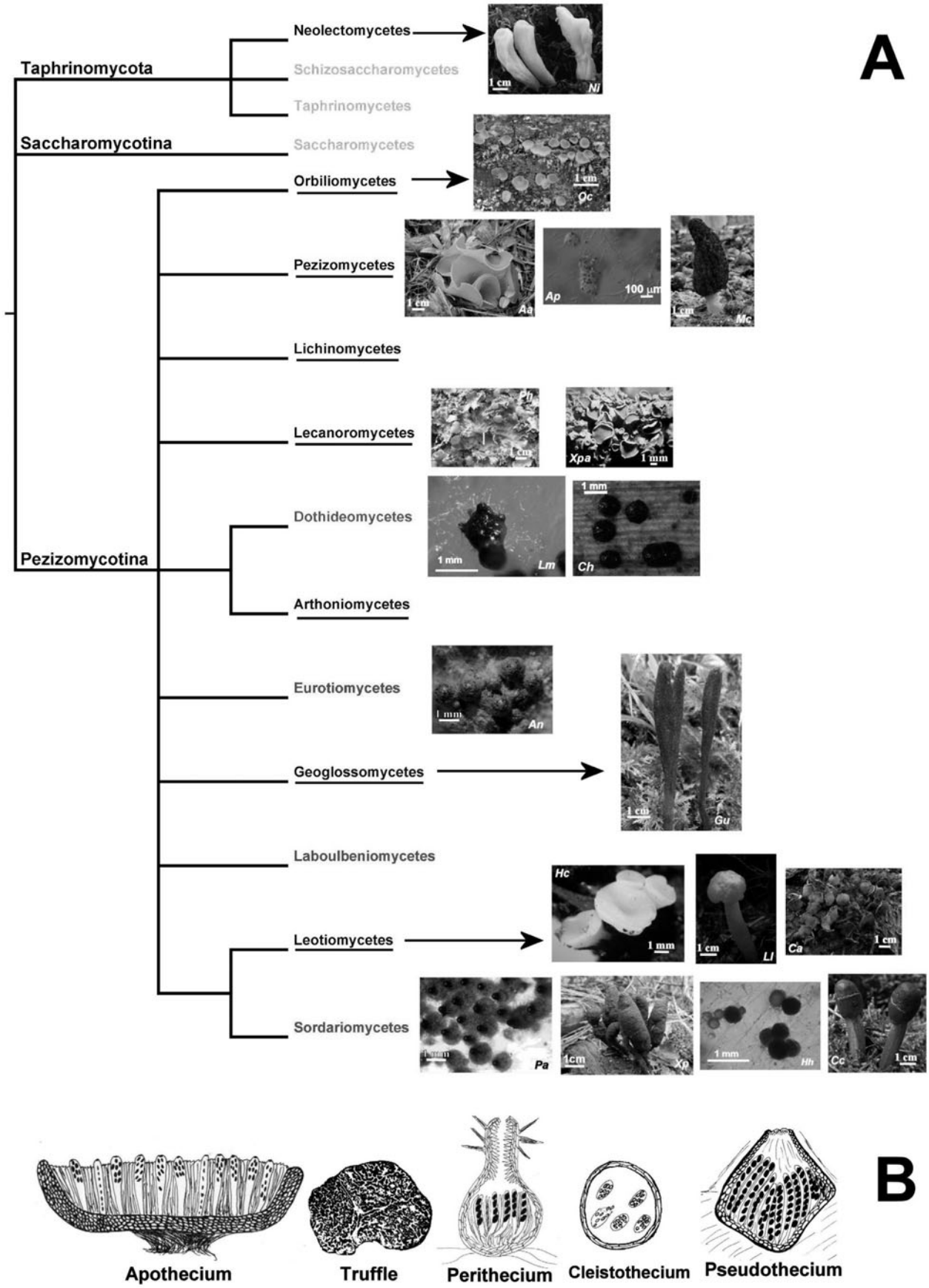
Multicellular fructifications of various shapes and ontogeny, called fruiting bodies, fructifications, ascocarps, or ascomata (singular, ascoma), are differentiated in Ascomycota, ensuring effective protection and dispersal of meiospores. Initial bouts of ancient evolutionary radiation in Ascomycota have resulted in present-day Taphrinomycotina, Saccharomycotina, and Pezizomycotina (Sugiyama et al., 2006) (Fig. 1A). Within each of these clades, further bouts of radiation have resulted in several independent evolutionary lineages (Spatafora et al., 2006; Sugiyama et al., 2006; Suh et al., 2006). One major characteristic differentiating these lineages is the ability or not to produce multicellular structures (Fig. 1A). Multicellular ascoma are not found in Saccharomycotina, but the members of at least one Taphrinomycotina lineage, Neolectomycetes, build simple but clearly multicellular fructifications. All lineages of Pezizomycotina generally produce more or less complex fruiting bodies (e.g., composed of several differentiated cell types). Interestingly, Pezizomycotina, like Basidiomycota able to produce multicellular fruiting bodies, can undergo anastomosis and have complex septal pores, both features ensuring effective communication between parts of the thallus. On the contrary, Saccharomycotina and Taphrinomycotina (with maybe the exception of *Neolecta*) do not engage in anastomosis, have no or simple septal pores, and do not form multicellular fructification, three features that they share with lower fungi (i.e., Chytridiomycetes and Zygomycetes) not able to build multicellular fruiting bodies. Moreover, mutants of *N. crassa* and *S. macrospora* affected in their ability to perform anastomosis or affected in factors associated with septal pores are not able to build fructifications (Xiang et al., 2002; Pandey et al., 2004; Fleissner et al., 2005; Rasmussen and Glass, 2005; Engh et al., 2007). Overall, these data suggest that the ability to differentiate multicellular fructification requires an integration of the mycelium that only higher fungi possess. These multicellular ascomata ensure efficient meiospore production and dispersal in air by various means and have thus been a major asset for land colonization.

In the Neolectomycetes, fructifications are a few centimeters high (Fig. 1A; *Ni*) and are composed of a stalk and a fertile head (Landvik et al., 2003). The cellular events that take place in the hymenium are peculiar to Neolectomycetes (Landvik et al., 2003), arguing for an independent appearance of multicellular fructifications in the lineage. However, no molecular analysis indicates whether the multicellular fructification development is the result of evolutionary convergence or reflects an ancient ability of the phylum Ascomycota that has been lost in some lineages.

Molecular phylogenies show that most filamentous ascomycetes belong to the subphylum Pezizomycotina (Spatafora et al., 2006). Most lineages from this group form multicellular fructifications that originate from the proliferation and aggregation of hyphae of maternal origin following or preceding differentiation of female gametes. Two major types of ascoma ontogeny are described in Pezizomycotina. In ascohymenials, the female gametangia form directly on the mycelium and then recruit neighboring hyphae. Therefore, a significant part of the fruiting body develops after fertilization. In ascoloculares, stromatic structures differentiate first, and then locules form inside these stroma. Female gametangia differentiate within these locules, and the remaining tissue forms the envelope of the fructification. In ascoloculares, fructifications develop before female gametangia and thus before fertilization. Ascohymenials produce mainly apothecia, truffles, perithecia, and cleistothecia (Fig. 1B). These types of fructification can be distinguished by the different exposure to the surrounding medium of the hymenium, i.e., of the cells that will undergo meiosis and thereafter differentiate into ascospores. Ascoloculares produce mostly pseudothecia (Fig. 1B).

Apothecia are cup- or cushion-like and are formed by the aggregation of hyphae supporting the layer of fertile cells. These cells have direct access to the surrounding environment and are mixed with various amounts of sterile tissues (the hamathecium) that likely play a role in properly orienting the asci, as ascospores are forcibly discharged due to some turgor pressure generated during the maturation of the ascocarp. As seen from their phylogenetic distribution (Fig. 1A), apothecia possibly represent the ancestral fructification state in Pezizomycotina. They come in various sizes and forms, from the minute cushion-like translucent fruiting bodies of *Ascodesmis* to the large stalked and complex edible ascomata of *Morchella*, i.e., morels (Fig. 1A; Oc, Aa, Ap, Mc, Ph, Gu, Hc, and Ll). In some lineages, apothecia have evolved into new forms, such as truffles, perithecia, or cleistothecia, to cite only the most common ones. Truffles are fundamentally apothecia

FIGURE 1 Fruiting body plan evolution in filamentous ascomycetes. (A) Representative fruiting bodies of major evolutionary lineages. Lineages in light gray do not produce multicellular fructifications; those underlined produce mostly apothecia or apothecia-like (e.g., truffles and others) fructifications. Laboulbeniomycetes and Sordariomycetes produce mostly perithecia, Dothideomycetes produce mostly pseudothecia, and Eurotiomycetes produce mostly cleistothecia and gymnothecia. Abbreviations: *Ni*, *Neolecta irregularis*; *Oc*, *Orbilia curvatispora*; *Aa*, *Aleuria aurantia*; *Ap*, *Ascodesmis porcina*; *Mc*, *Morchella conica*; *Ph*, *Peltigera horizontalis*; *Xpa*, *Xanthoria parietina*; *Lm*, *Leptosphaeria maculans*; *Ch*, *Cochliobolus horizontalis*; *An*, *Aspergillus nidulans*; *Gu*, *Geoglossum umbratile*; *Hc*, *Helotium citrinum*; *Ll*, *Leotia lumbrica*; *Ca*, *Chlorociboria aeruginascens*; *Pa*, *Podospora anserina*; *Xp*, *Xylaria polymorpha*; *Hh*, *Haematonectria haematococca*; *Cc*, *Cordiceps capitata*. *Aa*, *Ap*, *Mc*, *Hc*, *Hh*, *Ll*, *Pa*, *Hh*, *Xpa*, *Lm*, *Xp*, *Cc*, *Ca*, and *An* photos are by P. Silar; images of *Oc* and *Gu* were kindly provided by J. L. Cheype, that of *Ch* by B. G. Turgeon, and that of *Ni* by Raymond Boyer. (B) Schematic representation of various Pezizomycotina fruiting bodies.


A
Taphrinomycota
Saccharomycotina
Pezizomycotina
Neolectomycetes
Schizosaccharomycetes
Taphrinomycetes
Saccharomycetes
Orbiliomycetes
Pezizomycetes
Lichinomycetes
Lecanoromycetes
Dothideomycetes
Arthoniomycetes
Eurotiomycetes
Geoglossomycetes
Laboulbeniomycetes
Leotiomycetes
Sordariomycetes
Ni
Oc
Aa
Ap
Mc
Pli
Xpa
Lm
Ch
An
Gu
Hc
Ll
Ca
Pa
Xp
Hh
Cc
B
Apothecium
Truffle
Perithecium
Cleistothecium
Pseudothecium

that have closed upon themselves and that are produced within soil, their dispersal being mainly ensured by the animals that feed on them.

Perithecia (Fig. 1A; *Pa* and *Hh*) are flask-shaped fructifications of about 1 mm, in which the hymenium is enclosed. Fertile cells may be mixed with various amounts of sterile tissue that have a structural role. Most possess an ostiole that permits the spores to be ejected outside the ascoma. In some species, such as some xylariaceae or cordycipitaceae, perithecia are grouped and embedded in stromatic tissues, yielding relatively large fructifications that are several centimeters high (Fig. 1A; *Xp* and *Cc*).

Cleistothecia (Fig. 1A; *An*) are minute, completely closed fructifications without enclosed sterile tissue, in which asci are scattered. They are likely to have evolved through the simplification of apothecia. An even more simplified fructification, the gymnothecia, may be encountered in onygenalean fungi. Pseudothecia (Fig. 1A; *Ch*) resemble perithecia, but their ontogeny is different. The end result of evolution may also be the complete loss of sexual reproduction in some species, in which reproduction is often ensured exclusively by the production of asexual spores. Note that convergent changes to the fructification shape are very common in fungi and that only the major fructification types for each clade are indicated in Fig. 1A. For example, this has been demonstrated for the evolution of apothecia into truffles (Laessoe and Hansen, 2007), pseudothecia (Lumbsch and Huhndorf, 2007), or cleistothecia (Stchigel and Guarro, 2007). Truffle fungi are a good illustration of convergent evolution, as they are found not only in Ascomycota, but also in Basidiomycota and in *Endogone* and *Glomus*. In Ascomycota, several lineages of Eurotiomycetes and Pezizomycetes contain truffles. At least 15 Pezizomycete lineages have independently invented truffles, and even today there remain some intermediate forms between typical apothecia and truffles (Laessoe and Hansen, 2007).

Hymenium Organization and Ascospore Production

Contrary to the extravagant array of ascoma shapes and colors, the fertile tissue giving rise to ascospores follows a fairly uniform development (Fig. 2A). In heterothallic species, it starts by the fertilization of two genetically compatible, usually morphologically dissimilar, gametangia. The fusion of both gametangia yields a multinucleate cell with two genetically dissimilar nuclei: the dikaryon. Homothallic species can dispense with fertilization and proceed directly to the multinucleate "dikaryon." From then on, two genetically compatible nuclei associate in a special cell, the ascogenous hypha. This cell undergoes a few divisions before adopting a special form, the crozier, which divides in a manner similar to that of vegetative dikaryotic hyphae of basidiomycetes. These binucleate dikaryotic cells are typical of higher fungi (hence their name, Dikaryomycota). In the crozier, the division of the two nuclei is synchronous. Two nuclei stay in the apical portion of the crozier and are soon separated from the others by a special septum, which divides

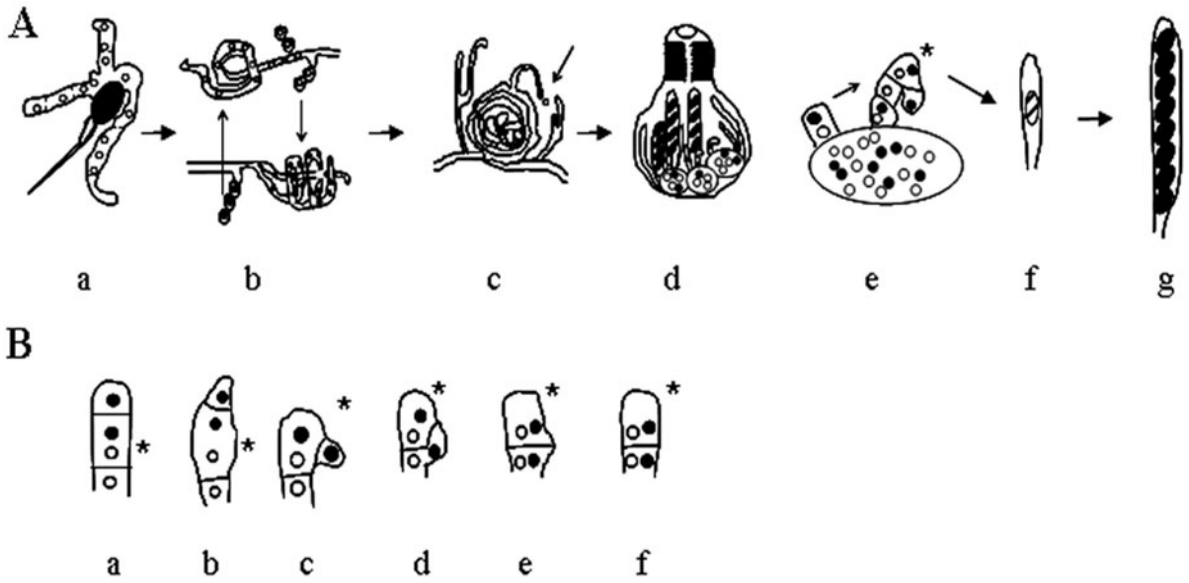


FIGURE 2 Life cycle and fruiting body development in Pezizomycotina. (A) Schematic representation of the major stages of the sexual cycle of a heterothallic pezizomycotina. The germinating ascospore (a) gives rise to a mycelium on which both male and female gametes differentiate (b). Fertilization occurs only between male and female gametes of opposite mating types (c). The trichogyne catches a microconidium (c, arrow), and the ascogonium develops into a perithecium (d). Inside the perithecium, dikaryotic ascogenous hyphae emerge from the plurinucleate dikaryotic cells. After one or two rounds of mitosis, the ascogenous hypha gives rise to a crozier (e). Karyogamy takes place in the upper cell (indicated by an asterisk) and is immediately followed by meiosis, a mitosis, and ascospore formation (f and g). (B) Schematic representation of various croziers (adapted from a drawing by M. Chadefaud [Chadefaud, 1960a]). All these structures derive from a dikaryotic hypha. The cell in which karyogamy takes place is indicated by an asterisk. (a through d) Tricellular crozier. (a and b) Simple crozier; (c) crozier with a lateral cell which does not fuse with the basal one; (d) prototypical crozier. The lateral cell will fuse with the basal one to give rise to another crozier cell; (e and f) bicellular crozier. The lateral cell does not exist any more.

the cell into three parts. This dikaryotic cell usually gives rise to an ascus, by undergoing first karyogamy, and then meiosis, and finally ascosporogenesis. In the prototypical crozier, the two remaining nuclei are left in two separate cells, which fuse together to yield a new dikaryotic cell, which in turn gives rise to a new crozier (Fig. 2A). The multiplicity of ascogenous hyphae and the successive generation of croziers are responsible for the high number of asci obtained after a single plasmogamy event. Other crozier forms, differing from the prototypical one, have been described by Chadefaud (Fig. 2B) (Chadefaud, 1960a). All these steps occur in the centrum, i.e., a defined region of the ascoma containing the fertile tissue intermixed with the sterile hamathecium.

Meiosis and postmeiotic mitoses are accompanied and followed by differentiation of the ascus and, within it, the ascospores. As for the fructifications, the shapes of the asci and ascospores vary from one species to another (Read and Beckett, 1996). Ascus morphology is often correlated with fungal classification, although quite a few evolutionary convergences are observed. Ascus walls may have different constitutions and present more or less complex opening systems to allow ascospore release. Unitunicate asci have a cell wall composed of layers that do not split in two and hence appear as a single layer under light microscopy. These asci are open at the apex by a special split or operculum in the operculate Pezizomycetes, whereas inoperculate Sordariomycetes, Leotiomycetes, and Lecanoromycetes have different apical apparatuses with complex morphologies. Cell walls of bitunicate asci split into two during maturation, allowing for special ascospore ejection. These cell walls are found mostly in the Dothideomycetes. Prototunicate asci possibly evolved by regression and are found mostly in Eurotiomycetes and in some Sordariomycetes. Their cell wall dissolves during maturation, so that ascospores cannot be forcibly discharged from the fruiting bodies. Ascospores may be uni- or pluricellular, hyaline or pigmented in various shades with melanin or other fungal pigments, minute or large, and with or without ornamentations. Ascospore differentiation has been used in the past for classification, but it is riddled, as is classification based on fructifications, with examples of convergent evolution. Thus, the development of the fructification wall has been described as a better predictor of species relatedness than ascospore forms in some members of the Sordariomycetes (Miller and Huhndorf, 2005), to cite but one example. After production, ascospores may be forcibly discharged from apothecia or perithecia or in contrast may await fructification dehiscence (cleistothecia) or ingestion by a mycophagous animal (truffle). Once released, their germination is also often subjected to complex environmental constraints, so that they germinate mainly in biotopes preferred by the fungus.

Coordination of Male and Female Gamete Differentiation Pathways

The reproductive structures are made in greater numbers than necessary, as only a fraction of them will succeed in participating in the sexual cycle. Several mutants affecting the number of male and female reproductive structures suggest that neither spermatium production nor female gametes are at their maximum level in wild-type strains. In *P. anserina*, null mutants of the *FLE1* gene, encoding a putative transcription factor with a C2H2 zinc finger similar to the FlbC protein of *A. nidulans*, are female hyperfertile but almost male sterile (Coppin, 2002). This antagonism led the author to suggest that the differentiation of female organs and spermatia are interconnected developmental pathways that may be in competition. This hypothesis is supported by the characteristics of *P. anserina incoloris* mutants. These mutants produce at least 1,000-fold more spermatia than wild-type strains, but no female organ. As observed for vegetative growth versus asexual sporulation or sexual reproduction, there seems to be a balance between the developmental pathways leading to male and female gamete formation. Identifying the genes located at the two *incoloris* loci in *P. anserina* (Bernet, 1986) and their relation to *FLE1* would be an interesting step in deciphering this balance.

MATING TYPES

Mating Systems

In addition to the morphological incompatibilities exhibited by the male and female gametangia, Pezizomycotina display genetic incompatibilities. Indeed, two reproductive styles, self-incompatibility (heterothallism) and self-compatibility (homothallism), are clearly distinguished in fungi. A self-incompatible individual is characterized by its ability to mate only with another individual from the same species, thus defining at least two compatible mating types. The compatibility between individuals of different mating types is controlled by a single genetic locus, a characteristic of self-incompatible ascomycota. This mating system was termed bipolar, as the progeny of a single fruiting body in a bipolar species are of two mating types. To date, all bipolar ascomycota have been found to harbor only two mating-type alleles, except for *Glomerella cingulata*, which is suspected to contain at least three mating-type alleles (Cisar and TeBeest, 1999).

Molecular analyses of the two-allele systems indicate that they correspond to different sequences occupying the same locus on their chromosome. These sequences were termed idiomorphs, a denomination proposed by Professor John Wyatt and popularized by R. L. Metzenberg and N. L. Glass (Metzenberg and Glass, 1990). There is yet no report of molecular characterization of a pezizomycotinan fungus in which mating is controlled by two genetic loci. This mating system is specifically found in Basidiomycotina (see chapter 34). However, genetic analysis of *Diaporthe perniciosa* suggests that this fungus might be tetrapolar, i.e., the progeny of a single fruiting body from this species displays four mating types (Cayley, 1931). Identifying a tetrapolar Ascomycota fungus would call for a reexamining of the currently accepted idea that tetrapolar mating evolved in Basidiomycota after their divergence from Ascomycota.

Self-compatible species are characterized by the ability of a single homokaryotic individual to yield a progeny, without any structural rearrangements to its mating-type locus. The mating types of self-compatible species are much more difficult to identify than those of self-incompatible species, as they do not behave as prominent genetic markers in a population. The term "idiomorph" is inappropriate for indicating the mating-type genes of self-compatible species, and we restrict the use of idiomorph to describe this locus in self-incompatible fungi.

Although most Pezizomycotina can be classified as self-compatible or self-incompatible, some species present peculiar mating characteristics. A first example of confusing lifestyle is provided by "pseudohomothallics." This lifestyle, known also as "secondary homothallism," is exemplified by *P. anserina* and *Neurospora tetrasperma*. Isolates of these species exist mainly as self-compatible individuals because their ascospores contain two nuclei of opposite mating types.

They can be resolved into homokaryotic self-incompatible cultures by fragmenting the mycelium or regenerating protoplasts, eventually revealing their unambiguous mating system. A second example of an unusual lifestyle is mating-type switching. Clonal individuals from these species are able to mate with themselves as self-compatible species. Nevertheless, this situation is different from true self-compatible reproduction, as a clonal individual becomes self-fertile because its mating-type locus undergoes a structural rearrangement. These individuals eventually contain two types of nuclei that are genetically different and sexually compatible (see "Mating Types in Switching Species" below). In contrast, *Sordaria brevicollis* offers a true case of a vanishing border between self-incompatible and self-compatible mating systems. *S. brevicollis* was the first recorded self-incompatible *Sordaria* species. It contains two mating types, *mta* and *mt*A, and the development of protoperithecia into perithecia normally occurs only when a culture is crossed with the opposite mating type. Surprisingly, *mt*A strains grown in the dark on cellophane disks produce fertile perithecia in the absence of the opposite mating type (Robertson et al., 1998). The mechanisms of this interesting mating system are still unclear, as *mt*A and *mta* have not yet been molecularly characterized. In some instances, self-compatible species mimic self-incompatibility by displaying a strong bias in favor of mating between two genetically different individuals. This phenomenon, termed "relative heterothallism," has been described in *A. nidulans* (Pontecorvo et al., 1953) and *Sordaria macrospora*. Heterokaryotic individuals containing nuclei with highly different genetic backgrounds produce almost exclusively recombinant progeny (Hoffmann et al., 2001). In contrast, heterokaryotic individuals containing nuclei that differ only at a single genetic marker produce a progeny in which one-half of the tetrads are identical to the parents. These data argue for a mechanism favoring sexual reproduction between different nuclei in a heterokaryon. This intriguing mechanism is thought to operate during fertilization (Hoffmann et al., 2001), but the role of mating types in relative heterothallism is yet unknown.

A survey of 10,596 ascomycetes showed that 55% may reproduce sexually, but evidence of mating is lacking for the remaining 45% of these species (Reynolds and Taylor, 1993). Whether or not these apparently asexual species ever undergo sexual reproduction or have cryptic or rare sex is often debated (reviewed by Taylor et al., 1999). Sharon et al. first found that *Bipolaris sacchari*, a fungus that has never been demonstrated to reproduce sexually, has idiomorphs similar to those of *C. heterostrophus*, a close relative with a self-incompatible mating system (Sharon et al., 1996). Subsequent searches of mating types in the supposedly asexual species have consistently confirmed their presence. Surprisingly, all asexual species that have undergone molecular characterization display a self-incompatible mating-type structure. They are consequently considered further in this review together with sexual self-incompatible species. All sequenced idiomorphs, with one exception (Yokoyama et al., 2006), provide no evidence of loss-of-function mutations in the mating-type genes, suggesting that these may still be functional and have not been subject to the pseudogene formation that would be predicted to occur if these species had evolved to be truly asexual. The potential for fertility of these genes was confirmed in some cases by the mating ability resulting from the replacement of idiomorphs from sexual species by idiomorphs of asexual close relatives. The recent discovery of a sexual cycle in *Aspergillus fumigatus* (O'Gorman et al., 2009) revives the question of a cryptic mating potential in asexual fungi. Dyer and Paoletti have reviewed the experimental findings of relevance to understand the mode of reproduction of a supposedly asexual species (Dyer and Paoletti, 2005). These findings combined molecular analyses of the diversity in population genotypes, the occurrence and frequency of mating types, the identification of genes involved in sexual reproduction in the genome, based on similarity to genes required for asexual cycle in *S. cerevisiae* and *Schizosaccharomyces pombe*, the transcription activity and pattern of these sex-related genes, and traces of a silencing process targeting duplicated sequences and acting during the fruiting-body formation. Unfortunately, these tests at best indicate that the species has undergone sexual reproduction recently but they do not prove that the species is currently sexually proficient. Dyer and Paoletti proposed the "slow decline" hypothesis to account for the loss of sexual proficiency (Dyer and Paoletti, 2005). This hypothesis is based on the observation that sexual species cultured for long periods in a vegetative state undergo a decline in sexual fertility. Such an effect is commonly observed during lab culture conditions and has been reported for *Histoplasma capsulatum* (Kwon-Chung et al., 1974; Fraser et al., 2007) and *Aspergillus glaucus* (Webster, 1980). However, it is likely that the demonstration that truly asexual fungi exist is just impossible, because it is impossible to demonstrate a negative result. The absence of mating types would not be a conclusive argument for the absence of a sexual cycle, because the genetics of reproduction appear to be more complicated than simple mating types. Mating-type genes are suspected to have lost their functions in some self-compatible species of the *Neurospora* genus (Wik et al., 2008), and nevertheless, sexual reproduction takes place in these species. On the contrary, neither karyogamy nor meiosis occurs in the ascogenous hyphae of the apomictic *Arnium arizonense* (*Podospora arizonensis*) (Mainwaring and Wilson, 1968; Mainwaring, 1971), although this self-compatible species contains mating-type genes that are counterparts of those of *P. anserina* (E. Coppin, D. Zickler, A. E. Bell, D. P. Mahoney, and R. Debuchy, unpublished data).

General Overview of Mating-Type Genes

Nomenclature

The proteins encoded by the mating-type genes of Ascomycota belong to two major categories: transcription factors and PPF proteins (see "MAT1-1-2" below). A sequence coding for a metallothionein-like protein has been identified in the mating-type locus of *Pyrenopeziza brassicae*, and, in a few cases, the idiomorph sequence expands and captures flanking genes. To date, no pheromone- or receptor-encoding genes have been found in the ascomycotan mating-type loci, in contrast to those of Basidiomycota (see chapter 34). Mating-type loci have a very different content among the Ascomycota, preventing a unified nomenclature in this group (Fig. 3). Mating-type loci in Saccharomycotina and Taphrinomycotina are characterized by the presence of homeodomain-encoding genes. In contrast, no homeobox gene has been found in the mating-type loci of Pezizomycotina. Transcription factors in Pezizomycotina, encoded by the mating-type locus, are related to either *S. cerevisiae* MATα1 or high-mobility group (HMG) proteins and constitute the basis of a standardized nomenclature.

The standardized nomenclature for idiomorphs and mating-type genes has been proposed, and in most cases adopted, to cope with the rapidly growing number of

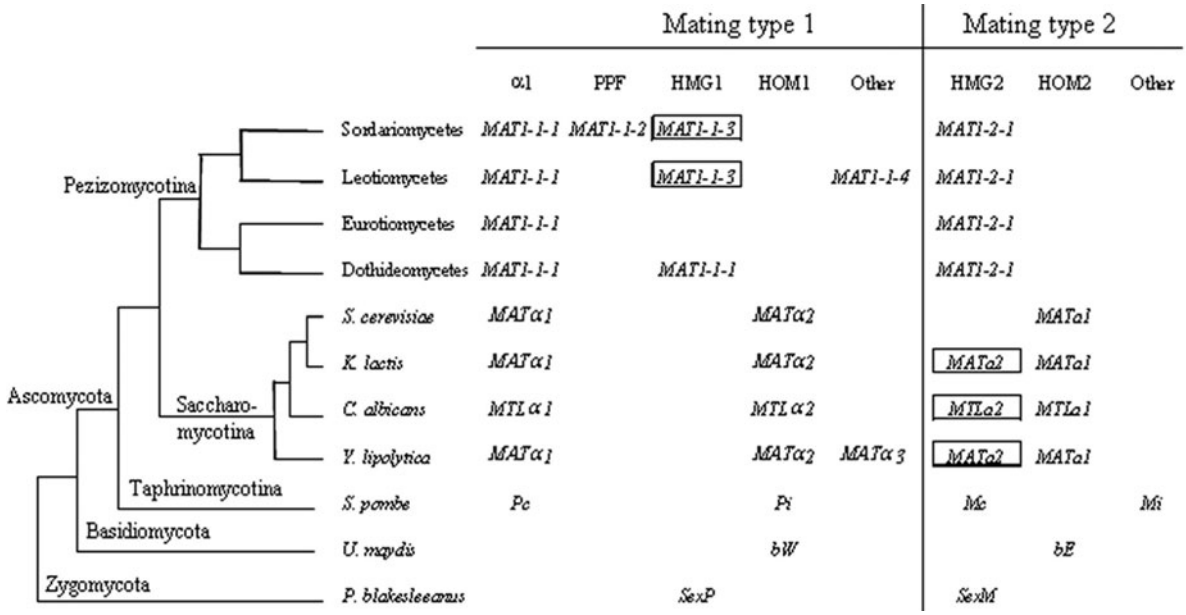


FIGURE 3 Mating-type structure in Fungi. α1, genes encoding transcription factors with an α1 domain; PPF, genes encoding proteins with a PPF domain; HMG1 and HMG2, genes encoding transcription factors with an HMG domain (phylogenetically related genes are boxed); HOM1, genes encoding transcription factor with a TALE homeodomain (reviewed by Bürglin, 2005); HOM2, genes encoding a transcription factor with a typical homeodomain; other, genes encoding proteins with uncharacterized features. Mating-type structures from *S. cerevisiae*, *Kluyveromyces lactis*, *Candida albicans*, and *Yarrowia lipolytica* were compiled from works of Butler and others (Butler et al., 2004; Butler, 2007). The mating-type structures of *S. pombe*, *Ustilago maydis*, and *Phycomyces blakesleeanus* were obtained from studies by Kelly et al. (1988), Kahmann et al. (1995), and Idnurm et al. (2008), respectively.

mating-type loci from various species (Turgeon and Yoder, 2000). To date, all self-incompatible Pezizomycotina, which have been genetically and molecularly analyzed, contain only one mating-type locus in accordance with their bipolar sexuality. This locus is *MAT1* in the standardized nomenclature. One idiomorph is characterized by the constant presence of a gene encoding a protein with a motif called the α1 domain (Fig. 3), found initially in the *S. cerevisiae* MATα1 transcription factor. This idiomorph is called *MAT1-1*, and the corresponding gene is *MAT1-1-1*. The other idiomorph is characterized by the invariant presence of a gene encoding a protein with a DNA-binding domain similar to that of the HMG (Fig. 3) and is called *MAT1-2*. The corresponding gene is called *MAT1-2-1*. The number assigned to any particular gene within an idiomorph corresponds to that of its homolog in other fungi, in which *MAT* genes have already been characterized. If there is no apparent homolog, the next sequential number is assigned. If a tetrapolar ascomycete were discovered, the two mating-type loci would be *MAT1* and *MAT2*. Designations in most fungal communities adhere to this system, with the exception of members of the *N. crassa* and *P. anserina* communities, as the designation of genes from these species was established long before the standard terminology was proposed.

MAT1-1-1

The α1 domain was initially identified by Glass et al. (Glass et al., 1990) in the *N. crassa* MATA-1 protein and subsequently found in all pezizomycotinan idiomorphs (Fig. 4). There are several studies supporting the role of MATα1 as a transcription factor in *S. cerevisiae* (reviewed by Herskowitz, 1989), but its three-dimensional structure has not yet been determined. Consequently, it is not known whether the α1 domain defines a new class of transcription factor or if it belongs to already-known sequence-specific DNA-binding protein families, such as those with basic domains that form dimers (helix-loop-helix), helix-turn-helix structures (homeodomain), and beta scaffolds (the HMG domain and proteins from the MCM1, Agamous, Deficiens, and Serum response factor family). In *S. cerevisiae*, MATα1 does not act alone to control mating and the expression of cell-type-specific genes. It forms a complex with MCM1 and STE12 to activate α1-domain-specific genes. Yuan et al. have identified a putative region of interaction with MCM1 in the α1 domain (Yuan et al., 1993). Interestingly, this region is localized in the most conserved region of the α1 domain in the MAT1-1-1 family (Fig. 4). Physical interactions between an MCM1 homolog and MAT1-1-1 have been demonstrated in *S. macrospora*, including the requirement of the MCM1 homolog in the production of fertile perithecia (Nolting and Poggeler, 2006). MAT1-1-1 proteins of *Cochliobolus* and relatives not only have an α1 domain but also have an HMG domain fused to, and located downstream from, the α1 domain (Metin et al., 2007; Turgeon and Debuchy, 2007). Turgeon et al. speculate that the fusion of the α1 and HMG domains may be functionally equivalent to the *MAT1-1* mating-type structure in Sordariomycetes, which contains a gene encoding the

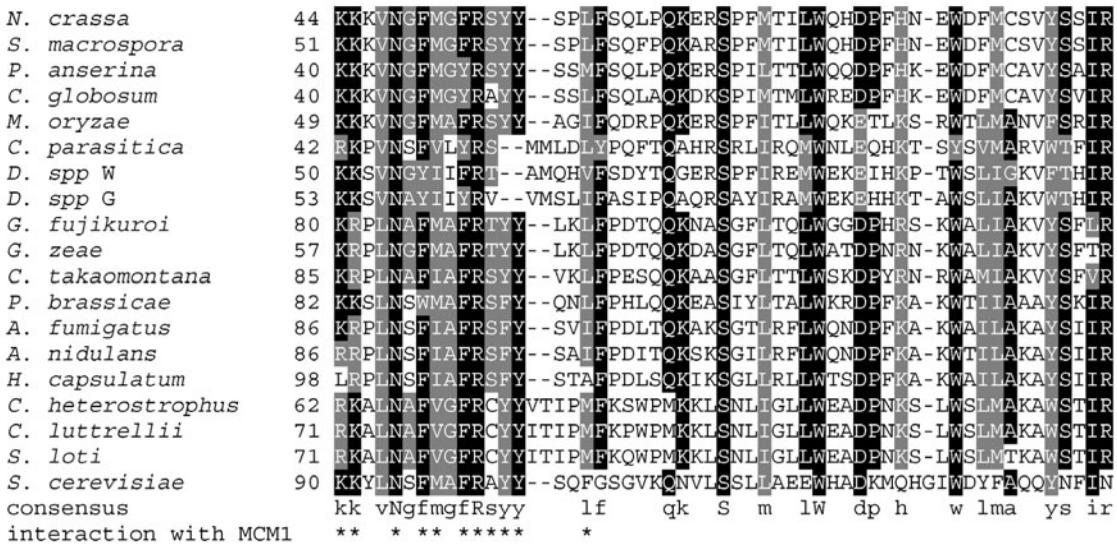


FIGURE 4 Amino acid alignment of the α1 domain of deduced MAT1-1-1 proteins of selected pezizomycotina with the MATα1 protein of *S. cerevisiae*: *N. crassa* (AAC37478), *S. macrospora* (CAA71623), *P. anserina* (CAA45519), *C. globosum* (EAQ89967), *M. oryzae* (strain 70-6) (BAC65087), *C. parasitica* (AAK83346), *Diaporthe* sp. W (BAE93750), *Diaporthe* sp. G (AB93756), *G. fujikuroi* (AAC71055), *G. zeae* (AAG42809), *C. takaomontana* (BAC67541), *P. brassicae* (CAA06844), *A. fumigatus* (AAX83122), *A. nidulans* (EAA63189), *H. capsulatum* (AB087596), *C. heterostrophus* (CAA48465), *C. luttrellii* (AAD33439), *Stemphylium loti* (AAR04470), and *S. cerevisiae* (P01365). Stars indicate the residues of the α1 proteins that may interact with MCM1 (adapted from Yuan et al., 1993). Fraction that must agree for shading is 0.7. Alignment was performed with Clustal version 2 (Larkin et al., 2007), and shading was done with BOXSHADE version 3.21.

HMG domain in addition to the *MAT1-1-1* gene (Fig. 3). This speculation is supported by the genetic and physical interaction data provided by the *P. anserina* mating system. In this fungus, the domains encoded by the *C. heterostrophus MAT1-1-1* gene are present in *FMR1* (α1 box) and *SMR2* (HMG box) genes. These two *mat* genes are involved in fertilization and are required for the formation of biparental ascogenous hyphae (reviewed by Turgeon and Debuchy, 2007). Moreover, the proteins encoded by *FMR1* and *SMR2* were found to interact in yeast two-hybrid assays (Arnaise et al., 1995). Together, these data indicate that the α1 and HMG proteins from the same idiomorph cooperate and interact physically throughout the sexual cycle of *P anserina*. Further investigations will be necessary to confirm the function of the HMG domain of the MAT1-1-1 protein in Dothideomycetes. Demonstrating that *Cochliobolus* MAT1-1-1 consists of functional linked α1 and HMG domains would strongly support an ancestral relationship between these domains in Pezizomycotina and would raise the question of whether the ancestral form was the sordariomycete or dothideomycete structure. Eurotiomycetes have a mating-type structure identical to that of Dothideomycetes (Fig. 3), but their *MAT1-1-1* genes are yet to be analyzed for the presence of an HMG box. The presence of an HMG domain in the *MAT1-1-1* gene would substantiate the idea that the fused α1::HMG domain is ancestral.

MAT1-1-2

Alignment of the MAT1-1-2 proteins led to the initial determination of a conserved region called the HPG domain, based on the conservation of three invariant residues, histidine, proline, and glycine (Debuchy and Turgeon, 2006). This alignment was reexamined by Kanematsu et al., who added four species from the genus *Diaporthe* and failed to find the HPG domain (Kanematsu et al., 2007). However, these authors succeeded in identifying three new invariant residues, two prolines and one phenylalanine, in the conserved region (Fig. 5). Accordingly, the name of this region has been changed to PPF. Surprisingly, mutations of these three conserved residues to alanine in the *SMR1* gene of *P. anserina* does not affect the sexual cycle, indicating that their conservation is not critical for the function of this protein (Coppin et al., 2005a). Extensive alanine scanning and missense and nonsense mutations throughout SMR1 revealed only two mutations that result in a complete arrest of the sexual cycle after fertilization: the change of tryptophan 193 and alanine 347 to alanine and aspartic acid, respectively (E. Coppin, unpublished results). Based on the high isoelectric point of the conserved domain of SMR1, Debuchy et al. have suggested that it defines a new DNA-binding domain (Debuchy et al., 1993).

No further progress has been made on the molecular function of MAT1-1-2, which remains unknown. However, *MAT1-1-2* knockout in *P. anserina* (reviewed by Turgeon and Debuchy, 2007), *S. macrospora* (Klix and Poggeler, 2008), and *G. zeae* (M. Dufresne, C. Vasnier, E. Coppin, D. Zickler, and R. Debuchy, unpublished results) led to a complete arrest of the fruiting-body development before ascogenous hyphae formation, indicating that this protein play a critical role in the sexual cycle in Sordariomycetes. A somewhat similar but leaky phenotype, consisting of infrequent ascogenous hyphae, was observed in *N. crassa*

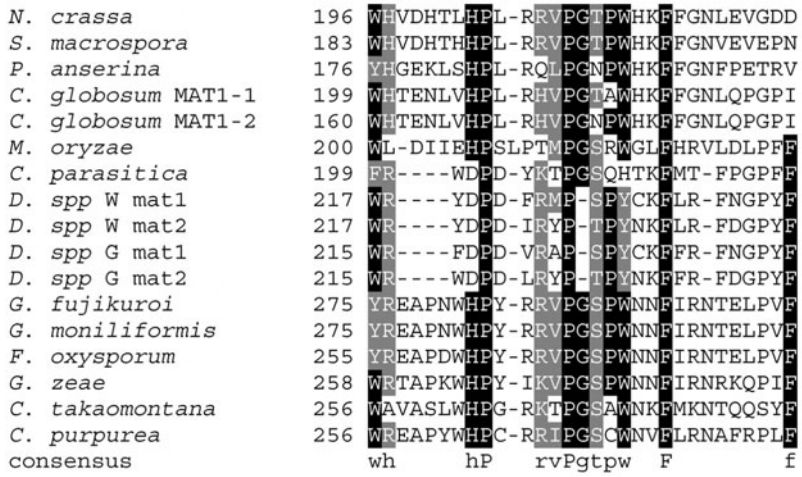


FIGURE 5 Amino acid alignment of the PPF domain of deduced MAT1-1-2 proteins of various pezizomycotina: *N. crassa* (AAC37477), *S. macrospora* (CAA71626), *P. anserina* (CAA52052), *C. globosum* MAT1-1 (EAQ89966), *C. globosum* MAT1-2 (EAQ91646), *M. oryzae* (strain 70-6) (BAC65088), *C. parasitica* (AAK83345), *Diaporthe* sp. W mat1 (BAE93749), *Diaporthe* sp. W mat2 (BAE93752), *Diaporthe* sp. G mat1 (BAE93755), *Diaporthe* sp. G mat2 (BAE93758), *G. fujikuroi* (AAC71054), *F. oxysporum* ((Yun et al., 2000), *G. zeae* (AAG42811), *C. takaomontana* (BAC67540), and *C. purpurea* (BAD72603). Fraction that must agree for shading is 0.7. Alignment was performed with Clustal version 2 (Larkin et al., 2007), and shading was done with BOXSHADE version 3.21.

mat A-2 mat A-3 double mutant (Glass and Lee, 1992), while single mutants were not affected in their sexual cycle (Ferreira et al., 1998). This surprising result suggests that MATA-2 and MATA-3 are redundant and may function in the same pathway as the one controlled by SMR1/MAT1-1-2 in *P. anserina*, *S. macrospora*, and *G. zeae* (see also "Functions of Mating Types in Self-Incompatible Species" below).

MAT1-1-3 and MAT1-2-1

The MAT1-1-3 and MAT1-2-1 proteins contain a single HMG box and are presumably sequence-specific DNA-binding proteins. They belong to the MATA_HMG box group, which is included, with T cells (TCF) and Sry-related (SOX) transcription factors from animals, in the large MATA/TCF/SOX_HMG box family (Soullier et al., 1999). MAT1-1-3 and MAT1-2-1 proteins belong to the MATA family, but they have different features. MAT1-1-3 proteins lack the highly conserved consensus sequence PRkXseXrrR, which is present at the C-terminal end of the HMG domain in MAT1-2-1 proteins (Fig. 6 and 7). Phylogenic analysis of the MAT1-1-3 and MAT1-2-1 proteins from Sordariomycetes indicates that all MAT1-2-1 proteins form a clade with a 98% bootstrap support (Fig. 8). Thus, MAT1-2-1 proteins may form a family differing from the MAT1-1-3 family in its structural and phylogenic characteristics. A striking feature of these HMG proteins is the similarity of their most conserved region to

FIGURE 6 Amino acid alignment of the HMG domain of deduced MAT1-1-3 proteins of selected pezizomycotina. *N. crassa* (AAC37476), *P. anserina* (CAA52051), *C. globosum* (EAQ89965), M. *oryzae* (BAC65085), C. *parasitica* (AAK83344), *Diaporthe* sp. W mat1 (BAE93748), *Diaporthe* sp. W mat2 (BAE93751), *Diaporthe* sp. G mat1 (BAE93754), *Diaporthe* sp. G mat2 (BAE93757), G. *fujikuroi* (AAC71053), G. *zeae* (AAG42812), and *P. brassicae* (CAA06846). Fraction that must agree for shading is 0.7. Alignment was performed with Clustal version 2 (Larkin et al., 2007), and shading was done with BOXSHADE version 3.21.

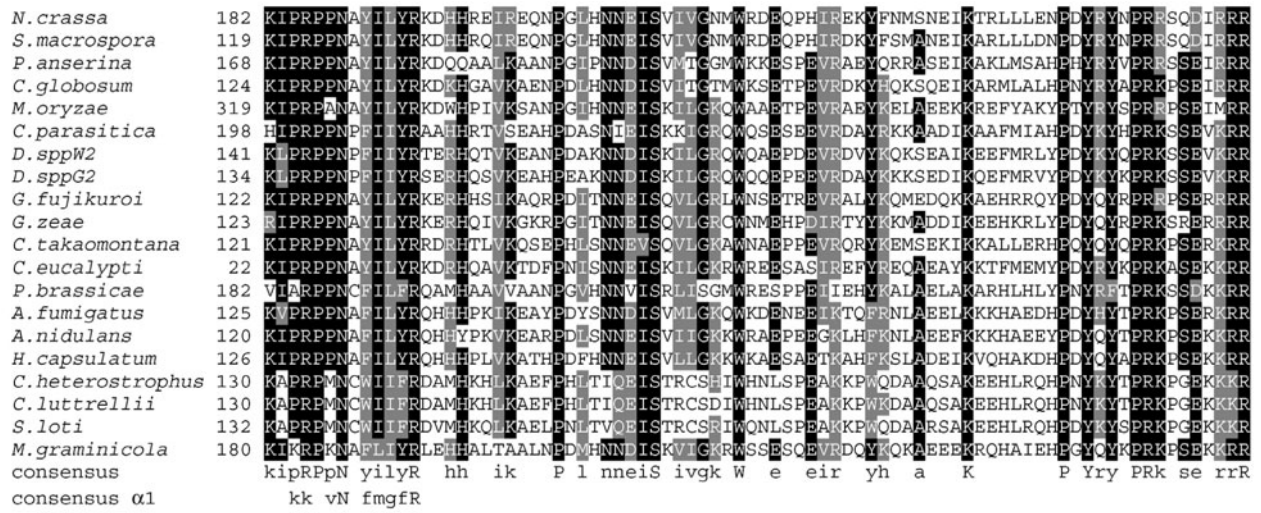


FIGURE 7 Amino acid alignment of the HMG domain of deduced MAT1-2-1 proteins of selected pezizomycotina: *N. crassa* (AAA33598), *S. macrospora* (CAA71624), *P. anserina* (CAA45520), *C. globosum* (EAQ91645), *M. oryzae* (BAC65090), *C. parasitica* (AAK83343), *Diaporthe* sp. W2 (BAE93753), *Diaporthe* sp. G2 (BAE93759), *G. fujikuroi* (AAC71056), *G. zeae* (AAG42810), *C. takaomontana* (BAC66503), *C. eucalypti* (AAF00498), *P. brassicae* (CAA06843), *A. fumigatus* (EAL92951), *A. nidulans* (AAQ07985), *H. capsulatum* (ABO87595), *C. heterostrophus* (CAA48464), *C. luttrellii* (AAD33439), *Stemphylium loti* (*S. loti*) (AAR04483), *M. graminicola* (AAL30836). Fraction that must agree for shading is 0.7. Alignment was performed with Clustal version 2 (Larkin et al., 2007), and shading was done with BOXSHADE version 3.21.

that of the α1 domain (Fig. 4, 6, and 7). This similarity suggests that the α1 domain is related to the HMG domain. We propose that the α1 domain might have evolved from an HMG domain in a protein containing a duplicated HMG region. This hypothesis would support the ancestral origin of the fused α1 and HMG domains of the *Cochliobolus* MAT1-1-1 protein. The three-dimensional structure of the α1 domain should provide key elements to determine its origin. Alternatively, as this conserved region in α1 proteins is thought to interact with MCM1 homologs, it is possible that the same region in HMG box proteins is also involved in the interaction with MCM1 homologs. It must be noted that in *S. cerevisiae* MCM1 is a transcriptional activator of both *a* and α-specific genes (reviewed by Herskowitz, 1989), suggesting that the MCM1 ortholog in Pezizomycotina may also be interacting with the MAT1-1 and MAT1-2 transcription factors for activating the transcription of their target genes.

Structures of Mating Types

Structures of Mating Types in Sordariomycetes

Self-Incompatible Sordariomycetes

The mating types of the self-incompatible members of Sordariomycetes are characterized by the presence of four types of genes: an α1 gene, two HMG genes, and a PPF gene (Fig. 3). The prototypical structure consists of three *MAT1-1* genes encoding α1, PPF, and HMG proteins and one *MAT1-2* gene encoding an HMG transcription factor. This structure is present in *N. crassa*, *P. anserina*, *Cryphonectria parasitica*, and *Gibberella fujikuroi* (Fig. 9). Many fungi for which there is no evidence of sexual cycle reproduction display this prototypical structure, for example *Fusarium oxysporum* f. sp. *lycopersici* (Yun et al., 2000). In this fungus, the mating-type genes are structurally indistinguishable from the functional genes present in the close relative *G. fujikuroi*. The mating-type content and structure display a remarkable fluidity, with several departures from the prototypical model. The most striking example is found in the *Diaporthe* spp., in which each idiomorph contains three genes (Kanematsu et al., 2007). The *MAT1-1* idiomorphs have sordariomycete prototypical structure, and the *MAT1-2* idiomorphs contain genes homologous to *MAT1-1-2* (PPF) and *MAT1-1-3* (HMG), in addition to the *MAT1-2-1* (HMG) gene (Fig. 9). The *Diaporthe* structure may represent the ancestral condition for mating-type locus structure in the Sordariomycetes (Kanematsu et al., 2007). According to this hypothesis, the sordariomycete prototypical structure results from the loss of both *MAT1-1-2* and *MAT1-1-3* genes from the ancestral *MAT1-2* idiomorph. This gene loss evolutionary model can be extended further to the *MAT1-1* idiomorph of *Magnaporthe oryzae* and *Cordyceps takaomontana*. In *M. oryzae*, the start of the MAT1-1-3 protein is located within the *MAT1-1* idiomorph, but the main part of its coding sequence is localized outside the *MAT1-1* idiomorph (Kanamori et al., 2007) (Fig. 9). *C. takaomontana* is a clavicipitaceous fungus displaying an atypical *MAT1-1* idiomorph, characterized by an absence of *MAT1-1-3* (Yokoyama et al., 2003) (Fig. 9). The loss of the *MAT1-1-3* gene might be in progress in *M. oryzae*, while it is completed in *C. takaomontana*, as well as in close relatives forming a monophyletic clade within the Clavicipitacea family (Yokoyama et al., 2006). There is evidence that two members of this clade, *C. takaomontana* (Yokoyama et al., 2005) and *Cordyceps militaris* (Shrestha et al., 2004), can reproduce sexually, indicating that the loss of *MAT1-1-3* does not result in the loss of fertility.

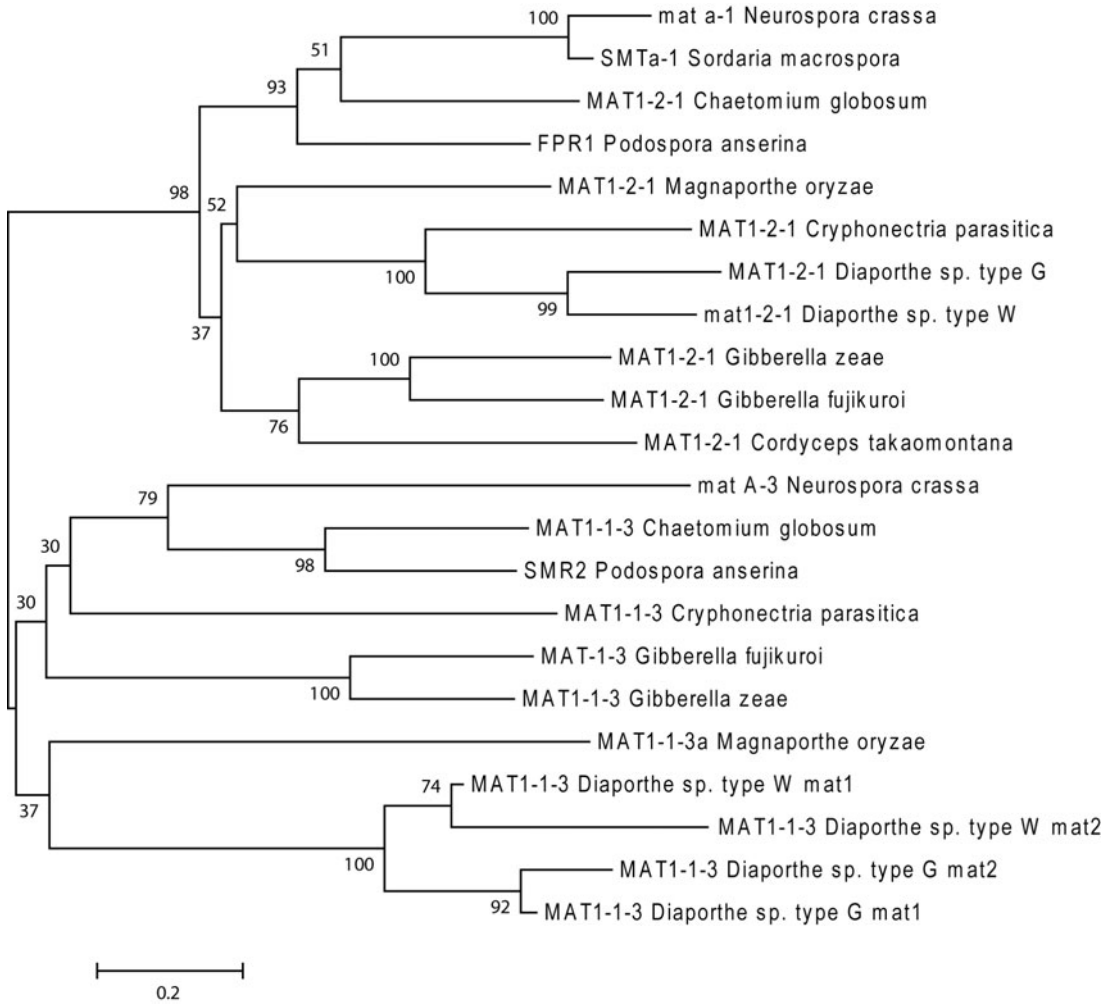


FIGURE 8 Phylogenetic tree of deduced MAT1-1-3 and MAT1-2-1 proteins of selected pezizomycotina. The neighbor-joining tree for the HMG proteins encoded by mating types indicates that MAT1-1-3 and MAT1-2-1 transcription factors form two distinct families. For the accession numbers of proteins, see Fig. 6 and 7. Phylogenetic analysis was conducted with MEGA 4 (Tamura et al., 2007).

Self-Compatible Sordariomycetes

Mating-type gene structure in self-compatible members of the Sordariomycetes has been determined for three species: *G. zeae*, *S. macrospora*, and *Chaetomium globosum* (Fig. 10). *G. zeae* carries linked counterparts of the *MAT* genes of self-incompatible sordariomycetes, i.e., three genes structurally identical to *MAT1-1* mating-type gene sequences in *G. fujikuroi*, separated from *MAT1-2-1* by 611 bp (Yun et al., 2000). *S. macrospora* contains a homolog of *MAT1-1-1* (called *SmtA-1*) and *MAT1-1-2* (called *SmtA-2*), whereas the counterpart of *MAT1-1-3* (called *SmtA-3*) lacks the region encoding the HMG domain (Pöggeler et al., 1997). *SmtA-3* is located 813 bp upstream from the *MAT1-2-1* gene (called *Smta-1*) and is cotranscribed with *Smta-1* (Pöggeler and Kück, 2000). The functional relevance of this unique case of cotranscription in filamentous fungi is unknown. The recently sequenced self-compatible *C. globosum* is phylogenetically closely related to *P. anserina* (James et al., 2006). It contains a *MAT1-1* sequence structurally identical to the *P. anserina mat–* idiomorph and a *MAT1-2-1* gene on a different supercontig (Debuchy and Turgeon, 2006). There is a perfect synteny between the *P. anserina mat* and the *C. globosum MAT1-1* environment. In contrast, the *C. globosum MAT1-2-1* environment displays no synteny with the *P. anserina mat*$^{+}$ environment, i.e., it does not contain any genes similar to *APC5*, *APN2*, *COX13*, or *SLA2* (Debuchy and Turgeon, 2006). Surprisingly, a gene similar to *MAT1-1-2* is located 1,540 bp upstream from *MAT1-2-1*. No model has been proposed for the origin of the *MAT1-2* arrangement, although the presence of duplications and transposases in the MAT1-2 environment might suggest that incorporation of this locus was mediated by transposons. Further analysis is necessary to explain the complete absence of synteny between the *C. globosum MAT1-2* locus and the sordariomycete mating-type region.

The mating-type structures of self-compatible species related to *N. crassa* have been explored by hybridization with cloned portions of the *N. crassa* idiomorphs (Glass et al., 1990; Glass and Lee, 1992; Beatty et al., 1994; Glass and Smith, 1994). In a subsequent analysis of the mating types in these self-compatible relatives of *N. crassa*, Wik et al. amplified each mating-type gene by PCR and analyzed its

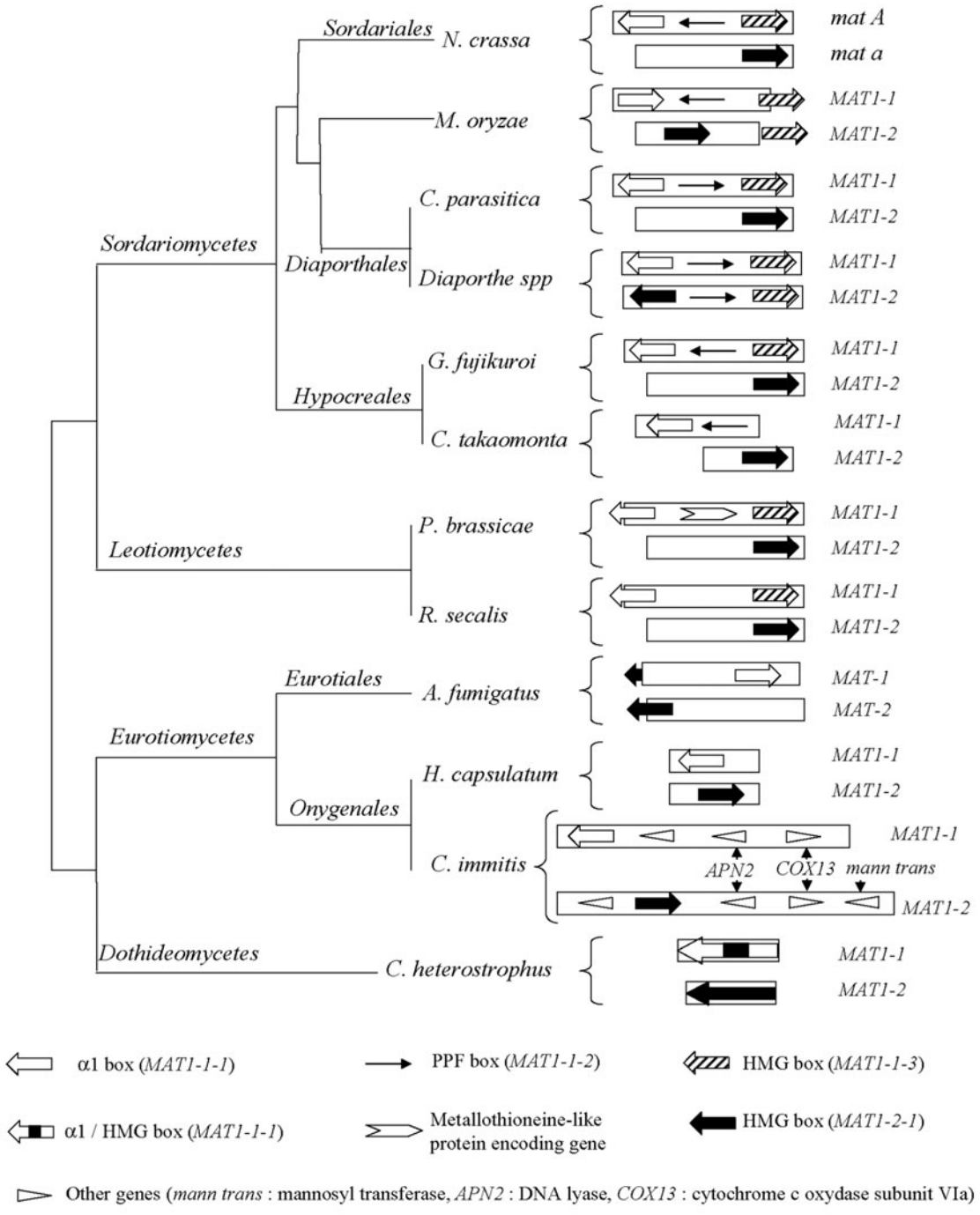


FIGURE 9 Structure of MAT locus in selected self-incompatible and asexual pezizomycotina. The phylogenic tree has been built upon previous studies by James et al. (2006). Mating-type locus structures are not to scale.

sequence (Wik et al., 2008). Both studies revealed three classes of self-compatible species. The first class contains the four mating-type genes that are present in prototypical mating types. The mating-type genes of this first class can potentially produce active proteins. This class includes *Gelasinospora calospora*, *Gelasinospora cerealis*, *Neurospora pannonica*, and *Neurospora sublineolata*. Wik et al. (Wik et al., 2008) report that the C termini of *MAT1-2-1* genes contain several mutations but the HMG domain is intact. The second group, exemplified by *N. terricola*, contains idiomorphs *MAT1-1* and *MAT1-2*, but the *MAT1-1-3* gene is missing. The third class, illustrated by *Neurospora* species *N. africana*, *N. dodgei*, *N. galapagosensis*, and *N. lineolata*, is characterized by the absence of the *MAT1-2-1* gene. Robertson et al. have suggested that the functions of MAT1-2-1 can be fulfilled by the HMG protein encoded by

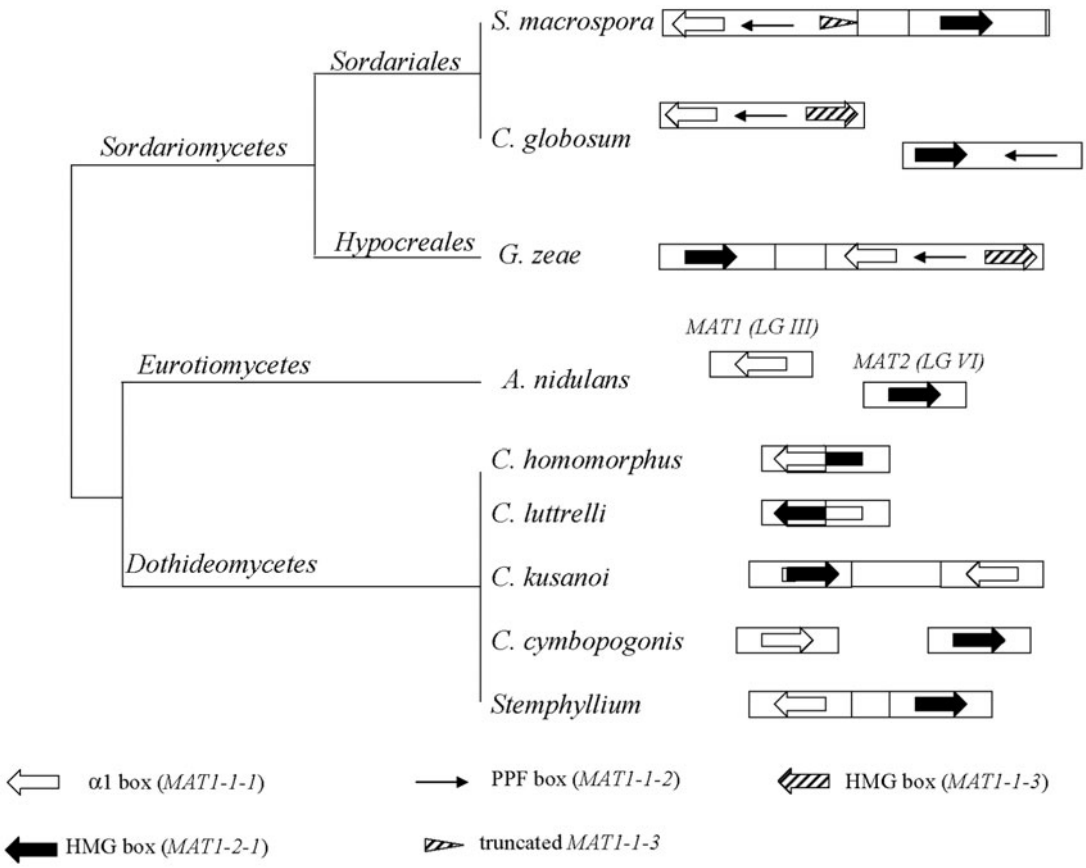


FIGURE 10 Structure of *MAT* locus in self-compatible pezizomycotina. The phylogenic tree has been built upon previous studies by James et al. (2006). Mating-type locus structures are not to scale.

MAT1-1-3 (Robertson et al., 1998). However, the region spanning the first half of the MAT1-1-3 HMG domain has been deleted in *N. africana* and *N. galapagosensis*, probably inactivating the HMG domain. Alternatively, Wik et al. suggest that the function of *MAT1-2-1* might be redundant with another HMG box gene in the genomes of these species (Wik et al., 2008). Several lines of evidence suggest that the *MAT1-1-2* gene is not active in *N. dodgei* and *N. africana*. Thus, the only gene that possibly remains functional in these species is *MAT1-1-1*. The *N. africana* *MAT1-1-1* gene induces fertilization in *N. crassa* (Glass and Smith, 1994), confirming that it is still functional, but further work is necessary to determine if *MAT1-1-1* and the other mating-type genes really have any function in mating in these self-compatible species.

Structures of Mating Types in Self-Incompatible Leotiomycetes

Two species from the self-incompatible Leotiomycetes, *P. brassicae* and *Rhynchosporium secalis*, have been analyzed for their mating-type structure: both lack the *MAT1-1-2* gene, but they contain HMG and α1 encoding genes in the *MAT1-1* idiomorph and an HMG box gene in the *MAT1-2* idiomorph (Fig. 9). Another common feature of these two fungi is that the coding region of the α1 box gene extends into the nonidiomorphic region. *P. brassicae* contains a new gene instead of *MAT1-1-2* (Singh and Ashby, 1998). This new gene, designated *MAT1-1-4* in agreement with the standard nomenclature, encodes a protein showing a high degree of similarity to metallothionein proteins. The authors suggest that this putative metallothionein-like protein functions as an environmental sensor for triggering sexual morphogenesis when the host plants become senescent and accumulate more metal ions. *R. secalis* is a fungus closely related to *P. brassicae*, based on internal transcribed spacer sequences (Goodwin, 2002). However, in contrast to *P. brassicae*, there is no proof of a sexual cycle in *R. secalis* (Gilles et al., 2001). The *R. secalis* idiomorphs resemble those of *P. brassicae*. Surprisingly, no *MAT1-1-4* gene was identified between *MAT1-1-1* and *MAT1-1-3*, even though the intergenic sequence has a 65% identity with the corresponding region of *P. brassicae* (Foster and Fitt, 2003). It is not yet known whether the absence of *MAT1-1-4* in *R. secalis* might be related to the loss of a sexual reproductive cycle.

Structures of Mating Types in Eurotiomycetes

Self-Incompatible Eurotiomycetes

Aspergilli constitute the major group of self-incompatible Eurotiomycetes, but no mating-type structure has yet been reported for any self-incompatible member of this genus, although four heterothallic species from *Aspergillus* or closely related *Neosartorya* are known (reviewed by Dyer, 2007). The genomes of three asexual aspergilli of key importance in

the medical, food, and biotechnology sectors have been sequenced and searched for mating-type genes. The in-depth analysis of the genome and population structure in *A. fumigatus* revealed several key characteristics of a sexual species (reviewed by Dyer and Paoletti, 2005). The presence of a fully functional reproductive cycle was confirmed recently (O'Gorman et al., 2009). The *A. fumigatus* isolates contain either the *MAT1-1* or *MAT1-2* idiomorphs, encoding an α1 or a HMG domain protein, respectively (Fig. 9) (Pöggeler, 2002; Varga, 2003; Galagan et al., 2005; Paoletti et al., 2005). Comparison of the *MAT1-1* and *MAT1-2* idiomorphs reveals that although the coding sequence of the HMG-box gene commences within the *MAT1-2* idiomorph region, the final 380 bp of the HMG box gene are localized within the nonidiomorphic region common to both *MAT1-1* and *MAT1-2* isolates (Fig. 9). The sequenced genome of *Aspergillus oryzae* contained an α1 gene (Machida et al., 2005). The additional HMG locus was identified by a population survey, which revealed the presence of *MAT1-1* and *MAT1-2* idiomorphic forms (P. Dyer, M. Paoletti, D. B. Archer, N. Yamamoto, and K. Kitamoto, personal communication). Crosses can now be set up to determine whether it is possible to induce sexual reproduction in vitro in this supposed asexual species. The genome of an industrial strain of the asexual species *A. niger* also contains an α1 box (Pel et al., 2007), but an idiomorphic arrangement has not yet been reported in the population. The analysis of the genome also reveals a premature stop codon in the ortholog of the *S. macrospora pro1* gene. This gene is required in *S. macrospora* for the transition from the protoperithecial to the perithecial stage (Masloff et al., 1999), suggesting that the sequenced *A. oryzae* may actually be asexual, at the very least as a result of this mutation.

Members of the Onygenales include a number of dimorphic primary pathogens, including *H. capsulatum*, *Coccidioides immitis*, and *Coccidioides posadasii*. While sexuality is uncommon among pathogenic fungi, *H. capsulatum* is one of the exceptions to this generalization. The structure of its *MAT* locus consists of two idiomorphs, each one encoding an α1 or an HMG domain protein and very similar in structure to those of Dothideomycetes (Fig. 9) (Fraser et al., 2007). In contrast to *H. capsulatum*, sexual reproduction by *C. immitis* and *C. posadasii* has never been observed, but population genetics studies suggest that recombination occurs. Genome sequencing and bioinformatics analysis has allowed several groups to identify the *MAT* locus (Bubnick and Smulian, 2007; Fraser et al., 2007; Mandel et al., 2007). Structurally, *Coccidioides* idiomorphs are the largest reported for ascomycetes, the *MAT1-1* and the *MAT1-2* idiomorphs being 8 and 9 kb in length, respectively. The *MAT1-1* idiomorph contains four genes, and the *MAT1-2* idiomorph has five genes (Fig. 9), more than the number observed in any other member of the Pezizomycotina. The roles of the additional genes in the *Coccidioides MAT* loci during sexual reproduction are not known. Mandel et al. suggested that *Coccidioides* expanded their *MAT* idiomorphs by the acquisition of two genes, *APN2* and *COX13*, frequently found adjacent to mating-type idiomorphs in the flanking regions common to both loci (Mandel et al., 2007). The identification of the mating types in *Coccidioides* sp. allowed Mandel et al. to analyze the distribution of the two mating types (Mandel et al., 2007). However the *Coccidioides* populations are analyzed (clinical or environmental isolates), the 1:1 distribution of *MAT1-1* and *MAT1-2* loci is indicative of a sexually reproducing species, supporting previous evidence of recombination.

Self-Compatible Eurotiomycetes

A. nidulans is the most representative species of self-compatible Eurotiomycetes. It contains unlinked counterparts of the *MAT1-2-1* and MAT1-1-1 genes (Dyer et al., 2003). These unlinked *MAT* genes were termed *MAT2* and *MAT1*, respectively, to recognize their respective locations on different chromosomes within the *A. nidulans* genome (Fig. 10). Homologs of the *MAT1-1-2* (PPF box) and *MAT1-1-3* (HMG box) genes found in the Sordariomycetes taxa do not exist in *A. nidulans* (Miller et al., 2005). *Neosartorya fischeri* has *MAT1* and *MAT2* genes that are unlinked (Rydholm et al., 2007), as those of *A. nidulans*. However, the organization of the mating-type environment seen in *N. fischeri* differs from that seen in *A. nidulans*, while *A. fumigatus* and *A. oryzae* share a conserved mating-type environment (Rydholm et al., 2007). This observation has led Rhydolm et al. to propose that heterothallism is the ancestral state of the section Fumigati and the aspergilli as a whole, while previous work seemed to confirm the general idea that homothallism is ancestral in this group (Galagan et al., 2005).

Structures of Mating Types in Dothideomycetes

Self-Incompatible Dothideomycetes

The *MAT* organization differs greatly within self-incompatible members of the Sordariomycetes, Leotiomycetes, and Eurotiomycetes; in contrast, the MAT loci of Dothideomycetes display a strikingly similar organization in all species examined to date. It consists of two idiomorphs, each harboring a single gene encoding a unique protein of the α1 or HMG type. Following standard nomenclature, described above, the genes have been designated *MAT1-1-1* and *MAT1-2-1*. The size of the idiomorphs varies with species, but the two encoded *MAT* genes have the same orientation with respect to their chromosome.

The *MAT* idiomorphs of *C. heterostrophus* are approximately 1.1 kb and are almost entirely occupied by the coding sequences (Turgeon et al., 1993) (Fig. 9). *C. heterostrophus* strains with the mating-type genes deleted (Wirsel et al., 1996) were used to test the functionality of cloned mating-type genes of asexual relatives. The heterologous expression of *Alternaria alternata* mating-type genes illustrates the ambiguous answers resulting from this approach. The expression of *A. alternata MAT1-1* and *MAT1-2* genes in separate *C. heterostrophus* strains (and mating of the strains) succeeded in inducing the formation of pseudothecia but failed to produce a progeny (Arie et al., 2000). Although *Alternaria* is a sister genus to *Cochliobolus*, the evolutionary distance between the two species may hamper effective expression and action of mating-type genes. Alternatively, the mating-type genes of *A. alternata* possibly lose their postfertilization activity, thus leading this species to lose sexual reproduction.

Phaeosphaeria nodorum is a wheat pathogen with a self-incompatible lifestyle. Its mating-type genes are clearly homologs of those examined in other Dothideomycetes, yet the *P. nodorum* idiomorphs are larger (approximately 4.3 kb) than those of *C. heterostrophus* (Bennett et al., 2003). *ORF1* is present on the common 5′ flanking region in *P. nodorum* and is also present upstream from *MAT* genes in other dothideomycetes related to *C. heterostrophus*. In contrast to the other species, only the 5′ section of the coding sequence of the *P. nodorum ORF1* ortholog is highly conserved. Degeneracy of conservation within the coding sequence signals the flank to idiomorph border (Bennett

et al., 2003). Similarly, idiomorphs in *A. alternata* extend toward *ORF1*, so there is only 4 bp between the *ORF1* stop codon and the idiomorph start (Bennett et al., 2003). It is likely that these species are expanding their *MAT* idiomorphs towards the acquisition of *ORF1*, a situation that is highly reminiscent of the expansion of *Coccidioides* spp. mating types (see "Structures of Mating Types in Eurotiomycetes" above).

Mycosphaerella graminicola is a self-incompatible dothideomycete and is the major cause of wheat disease in northwestern Europe. Based on the collection of ascospores, Kema et al. demonstrated that *M. graminicola* undergoes a sexual cycle from spring to autumn (Kema et al., 1996). *M. graminicola* contains *MAT1-1* and *MAT1-2* idiomorphs resembling those of *C. heterostrophus*, but the *M. graminicola* idiomorphs are larger (approximately 2.8 kb) (Waalwijk et al., 2002). Phylogenetic trees place the MAT1-1-1 and MAT1-2-1 proteins of *M. graminicola* closer to their homologs in Leotiomycetes and Sordariomycetes than to MAT1-1-1 and MAT1-2-1 of *C. heterostrophus*. Accordingly, *APN2* and *APC5* genes are present in close vicinity of the mating-type locus of *M. graminicola*, Sordariomycetes, and Leotiomycetes, but none of these genes are present in the environment of the mating-type locus of *C. heterostrophus* (Wirsel et al., 1998), *A. alternata* (Bennett et al., 2003), and *Ascochyta rabiei* (Barve et al., 2003). For instance, *ORF1* is the first gene present upstream from *MAT* genes in *C. heterostrophus*, whereas *ORF1* is absent from the idiomorphic environment in *M. graminicola*, which contains *APN2* upstream from its *MAT* genes (Waalwijk et al., 2002). These observations question the position of *M. graminicola* in the Dothideomycetes group, which may be polyphyletic, or the origin of the mating genes in the group of *Mycosphaerella*.

Self-Compatible Dothideomycetes

In stark contrast to the identical structural organization of *MAT* loci observed in self-incompatible Dothideomycetes, self-compatible representatives of this taxon display all possible arrangements of the two mating-type genes found in self-incompatible species. Yun et al. have examined the arrangement of mating-type genes in self-compatible *Cochliobolus* species (Yun et al., 1999). In *C. luttrelli* and *C. homomorphus*, the *MAT1-1-1* and *MAT1-2-1* genes with their α and HMG boxes are fused into a single coding sequence; the gene order in *C. luttrelli* is reversed in *C. homomorphus* (Fig. 10). Yun et al. have suggested that a crossover event occurred within the dissimilar mating-type genes of a self-incompatible ancestor at positions corresponding to the fusion junction. In *C. kusanoi*, an intact counterpart of the *MAT1-1-1* gene is present 1,092 bp downstream of *MAT1-2-1* (Fig. 10). *C. cymbopogonis* carries both homologs of the self-incompatible idiomorphs, but these are not closely linked. For the last two species, the mechanism leading to the observed arrangement remains unknown.

Stemphylium is a genus closely related to *Cochliobolus*, suggesting that the mating system of the two genera might have evolved in similar ways. The analysis of 85 self-fertile isolates of *Stemphylium* spp. indicates that 76 isolates contain an inverted *MAT1-1-1* gene less than 1.5 kb from a *MAT1-2-1* gene (Fig. 10), whereas only *MAT1-1* was detected in the remaining seven isolates (Inderbitzin et al., 2005), a situation analogous to what is observed for *N. africana*, *N. dodgei*, *N. galapagosensis*, and *N. lineolata* (see "Structures of Mating Types in Sordariomycetes" above). The sequence analysis of the divergently transcribed *MAT* genes suggests that this structure may have originated from a self-incompatible ancestor by the inversion of an ancestral *MAT1-1-1* gene plus flanking sequences, creating a crossover site allowing the fusion of the inverted *MAT1-1* region to *MAT1-2*. The self-fertile species with fused mating-type regions are monophyletic in a mating-type region phylogeny but polyphyletic in a phylogeny based on four other loci. This contradiction can be solved by invoking a lateral transfer of the *MAT1-1* and *MAT1-2* arrangement across lineages in the evolution of *Stemphylium* (Inderbitzin et al., 2005). Therefore, the mating system in *Stemphylium* has evolved in a way very different from that in *Cochliobolus*, providing another example of the extraordinary fluidity of the mating-type genes.

Mating Types in Switching Species

In contrast to the stability of mating types in species such as those described above, what appears to be examples of mating-type switching has been described in some members of the Pezizomycotina. Little is known about mating-type switching in the Pezizomycotina, whereas it has been intensively studied in *S. cerevisiae* and *S. pombe* (reviewed by Lin and Heitman, 2007). The data gathered in studies of *Chromocrea spinulosa* (Mathieson, 1952), *Sclerotinia trifoliorum* (Uhm and Fujii, 1983), and many species of *Ceratocystis* sensu stricto (Harrington and McNew, 1997) suggest, however, that the switching mechanisms are different from those present in yeasts. The Pezizomycotina species are characterized by a population structure consisting of self-fertile and self-sterile individuals. Crosses of self-sterile with self-fertile isolates result in a 1:1 ratio of self-sterile to self-fertile progeny. Self-fertile isolates also yield progeny, which segregate 1:1, self-sterile to self-fertile. David D. Perkins first suggested that the production of self-sterile individuals in the progeny of a self-fertile isolate implies a unidirectional mating-type switch (Perkins, 1987). PCR analyses of the mating-type loci of *C. spinulosa* have been undertaken, using DNA from vegetative (nonmating) cultures of each type of *C. spinulosa* strains (S. H. Yun and B. G. Turgeon, unpublished data, quoted by Turgeon and Debuchy, 2007). Self-sterile strains all have the *MAT1-1* gene with the prototypical structure found in the Sordariomycetes (see "Structures of Mating Types in Sordariomycetes" above). Self-fertile strains have a *MAT1-1* gene, plus a second version of *MAT1-1* linked to a *MAT1-2-1* gene. The architecture of the version containing both *MAT* genes resembles that found in self-compatible sordariomycetes (Fig. 10). The presence of repeated sequences in the version with both *MAT* genes hints at an intramolecular recombination, whereby the *MAT1-2-1* gene might be eliminated, resulting in *MAT1-1* only. In *Ceratocystis* sp., Witthuhn et al. also found that the self-fertile isolates contain a *MAT1-2-1* gene that is lost in the self-sterile progeny (Witthuhn et al., 2000).

Molecular organization of mating types in *Ceratocystis coerulescens* is probably similar to that of *C. spinulosa*. Genetic analyses of the mating system suggest, however, that the *MAT1-1* mating type present in the so-called "self-fertile" individuals is in fact a silent copy, which is expressed only after the switch in mating-type and loss of *MAT1-2-1* (Harrington and McNew, 1997). This suggestion is supported by the fact that "self-fertile" *MAT1-2* strains are almost unable to fertilize *MAT1-2* strains by spermatization. Therefore, the "self-fertile" strains cannot function as *MAT1-1* individuals (Harrington and McNew, 1997) but instead behave as self-incompatible *MAT1-2*

individuals. Cultures of these strains were never seen to segregate self-incompatible sectors, which was interpreted as an indication that the switch in mating type occurs in ascogonia, perhaps before dikaryon formation (Webster and Butler, 1967; Harrington and McNew, 1997). Alternatively, the switch in mating type may take place in the mycelium, but most of the *MAT1-1* nucleus should quickly pair with a neighboring *MAT1-2* nucleus to form a fruiting body, thus preventing the formation of large *MAT1-1* clonal sectors in the mycelium. According to the latter suggestion, a few *MAT1-1* nuclei may participate in the formation of male cells and produce the very rare fertilization events observed when *MAT1-2* strains are used to spermatize *MAT1-2* tester strains (Harrington and McNew, 1997). The term "self-compatible" usually designating these strains is therefore misleading and should be employed cautiously. *C. spinulosa*, *S. trifoliorum*, and *Ceratocystis* spp. deserve more attention than they are currently given. Indeed, these organisms may provide another instance of the silencing mechanism thought to exist in self-compatible fungi (see "Shifting Reproductive Modes" below).

Botrytis cinerea is a bipolar self-incompatible fungus, displaying a Mendelian segregation of each mating type in random ascospore progeny. Nevertheless, strains sexually compatible with both reference strains of opposite mating types were recovered at a low frequency in the progeny. The analysis of ordered ascospore tetrads confirmed the Mendelian segregation of the self-incompatible phenotype in 98 asci examined and the appearance of the dual mater strains in 7 asci (Faretra and Pollastro, 1996). This new phenotype always replaces the same self-incompatible allele in two or four ascospores of the seven tetrads. The progeny obtained from pairing dual maters with a reference strain are sexually compatible with one or the other parent, except for 5% that behave as dual maters. There are no reports of any progeny obtained from self-fertilization of these dual maters, which therefore appear to be self-sterile. The molecular analysis of the mating types of *B. cinerea* should resolve this intriguing behavior, which might be attributed to the transient switch to a third mating-type allele conferring dual-mating ability, but not self-fertility.

Among the species displaying self-fertility and self-incompatibility is *G. cingulata*, a "sexually ambiguous species without peer" according to John R. Raper (Raper, 1966). Wheeler and McGahen report an extremely complicated pattern of sexuality in this species, resulting from the interaction of several genetic factors, some exhibiting high mutation rates (Wheeler and McGahen, 1952). Two multiallelic loci, *A* and *B*, are considered primarily responsible for the basic sexual characteristics, with more than 20 loci, unlinked to *A* and *B*, affecting the sexual process in some manner. David D. Perkins proposed that a high mutation rate from one allele of *B* to another results from a switch in mating type (Perkins, 1987). Cisar and TeBeest have analyzed the *G. cingulata* mating system and observed no switching; instead, they identified various threads of evidence supporting a mating-type locus with more than two alleles (Cisar and TeBeest, 1999). However, the *A* and *B* alleles of the strains used in this study were not known, preventing any correlation with the results reported by Wheeler.

Functions of Mating Types

The functions of mating-type genes have been investigated in a limited number of pezizomycotina. Studies of the self-incompatible organisms *P. anserina*, *N. crassa*, and *C. heterostrophus* point to MAT1-1-1 and MAT1-2-1 as the key regulatory factors for fertilization. There are several indications that the mating-type genes also control developmental events downstream of fertilization in these three fungi. A model for mating-type function has been proposed in *P. anserina* for internuclear recognition and the formation of biparental ascogenous hyphae, but it is unclear to what extent this model is applicable to other fungi. Strikingly, one mating-type gene, *SMR1/SmtA-2/MAT1-1-2*, appears to have the same biological role in *P. anserina*, in the self-compatible *S. macrospora*, and in *G. zeae* (see "MAT1-1-2" above). The mating-type genes of the self-compatible *S. macrospora*, *G. zeae*, and *A. nidulans* are undoubtedly required for the development of the fruiting body, but their particular functions are unknown. It must be emphasized that the authors suspect that mating-type genes may have no function at all in some self-compatible *Neurospora* species (Wik et al., 2008) (see "Structures of Mating Types in Sordariomycetes" above).

Functions of Mating Types in Self-Incompatible Species

Self-Incompatible Sordariomycetes

The functions of mating-type genes have been examined in depth in two fungi from the group of self-incompatible members of the Sordariomycetes: *N. crassa* and *P. anserina*. The model for mating-type function in *P. anserina* has been extensively reviewed recently (Turgeon and Debuchy, 2007). Briefly, mating-type genes control fertilization and the formation of biparental ascogenous hyphae. The *FPR1* transcription factor activates a set of genes that determine the *mat+* mating type, and it is also supposed to control a feature specific to *mat+* nuclei. This feature, termed nuclear identity, is assumed to direct the recognition between *mat+* and *mat−* nuclei, very much like mating types control the recognition between sexually compatible cells. This internuclear recognition is the first step in the formation of biparental ascogenous hyphae. Analyses of FPR1 mutations revealed that it also represses the functions that determine the *mat−* mating type and the *mat−* nuclear identity. The activator and repressor action of FPR1 is exemplified by the phenotype of mutations, which decrease its repressor activity on *mat−* functions without affecting its activating activity on *mat+* functions. The *mat+* strains expressing these mutated FPR1 regulatory proteins are weakly self-compatible, an indication that they no longer repress *mat−* functions. Critically, crosses of these mutants with a wild-type *mat−* tester strain give a progeny constituted of biparental and uniparental asci. All the latter asci only contain the *mat+* nucleus, indicating that the mutation in *FPR1* allows the *mat+* nuclei to self-recognize; this is probably because the *mat+* and *mat−* functions required for internuclear recognition are coexpressed in the *mat+* mutant nuclei. Equivalent mutations exist in *FMR1* and *SMR2*, leading to uniparental *mat−* progeny. Mutations in *SMR1* result in a complete arrest of the sexual development after fertilization and before the formation of ascogenous hyphae. Genetic relationships between mutations in *SMR1* and the three other mating-type genes suggest that crosses in the *SMR1* mutant context are blocked after internuclear recognition. This observation led the authors to suggest that internuclear recognition is associated with a developmental arrest that is overcome by the action of SMR1. The cause of the development arrest remains unknown. Wik et al. proposed that the function of *MAT1-1-2* genes may be related to mating-type-associated vegetative incompatibility (Wik et al., 2008), suggesting that the developmental arrest may result from an incompatibility reaction.

The *N. crassa* mating-type functions display considerable differences from those of the *P. anserina* system, even though these two fungi are closely related and possess identical mating-type organization. The *N. crassa* counterparts of *FMR1* (*mat A-1*) and *FPR1* (*mat a-1*) activate the A and *a* fertilization functions, respectively. However, no conclusive results support any repressor function of *mat A-1* on *a* functions or any repression of A functions by *mat a-1*. Although transcription of the α-type *ccg-4* pheromone gene in the *mat a-1*^{m33} mutant suggests that this mutant has lost a repressing function for *ccg-4*, *mat a-1*^{m33} mates as *mat a* (Bobrowicz et al., 2002), suggesting that the CCG-4 peptide is not produced and that additional regulators may control pheromone protein production and/or fertility. Moreover, the deletion of the mating-type genes does not induce the transcription of the pheromone genes (Bobrowicz et al., 2002). This observation invalidates the simple model of a repressing action of *mat a-1* on *ccg-4*. Strains with mutations in the counterparts of *SMR1* (*mat A-2*) or *SMR2* (*mat A-3*) are indistinguishable from a wild-type A strain (Ferreira et al., 1998), a striking difference with the *P. anserina* system. Surprisingly, a strain impaired in the expression of both *mat A-2* and *mat A-3* was drastically reduced in its capacity to produce ascospores (Glass and Lee, 1992). Analysis of perithecia obtained from this mutant showed that formation of ascogenous hyphae is infrequent, although occasional croziers can be observed among the paraphyses. The nuclei in these croziers undergo normal karyogamy and meiosis and form mature asci and viable ascospores. Taken together, these data indicate that *mat A-2* and *mat A-3* are redundant genes, a conclusion contradicting findings by Wik et al., who reported that *mat A-2* evolves under positive selection in *N. crassa* (Wik et al., 2008). Progress on mating-type function in *N. crassa* has been impeded for a long time, as mating-type gene ectopic copies fail to complement the resident mutated copies, leading to sexual cycle arrest (Glass et al., 1988). Subsequent discovery of meiotic silencing by unpaired DNA (MSUD), a process whereby an unpaired DNA fragment and all homologous copies are silenced during meiosis (Shiu et al., 2001), has highlighted this phenomenon. Although idiomorphs at their *MAT* locus are unpaired during meiosis, they are probably immune to silencing by MSUD, at least in their normal location. By contrast, ectopic copies of *mat a* or *mat A* idiomorphs trigger MSUD and result in barren perithecia. In harmony with this interpretation, a cross of *mat a* to the ectopic *mat A* strain was at least 100 times more fertile in a context defective for MSUD than the cross in a context of active silencing (Shiu et al., 2001). The action of MSUD on ectopic copies of the idiomorphs reveals that their information is required during meiosis, or shortly after this step, but the specific function of *mat* genes during meiosis remains to be established.

Self-Incompatible Dothideomycetes

In the Dothideomycetes group, mating-type gene function has been examined only in *C. heterostrophus* (reviewed by Turgeon and Debuchy, 2007). The sterility of strains in which the mating-type locus is deleted indicates that the *MAT* genes control fertilization (Wirsel et al., 1996), as observed in Sordariomycetes. Subsequent analysis of the 5′ and 3′ untranslated regions common to *MAT1-1* and *MAT1-2* genes revealed that a short deletion within the 3′ end of the transcript results in barren pseudothecia (Wirsel et al., 1998). The nature of this feature is unknown, but it must be present in both parents for the completion of the sexual cycle. This finding indicates unambiguously that the mating types of *C. heterostrophus* are required for developmental events following fertilization, in agreement with the conclusions on the role of mating-type genes in *P. anserina* and *N. crassa*.

Function of Mating-Type Loci in Self-Compatible Species

Self-Compatible Sordariomycetes

The deletion of the complete mating-type locus in *G. zeae* results in strains that produce perithecium-like structures, but these structures do not contain asci or ascospores, indicating that the *G. zeae* mating-type genes do have a critical role in sexual development (Desjardins et al., 2004). Deletion of either *MAT1-1* or *MAT1-2* sequences resulted in strains that form no perithecium-like structure, even after an extended incubation time, in contrast to the *MAT*-deleted strain (Lee et al., 2003). These differences can be interpreted with the *P. anserina* model of mating-type activation and repression. The tight phenotype may be attributed to the remaining *MAT* sequences, which may have a repressing activity on the opposite-mating-type target genes. In contrast, the complete deletion of the mating-type locus may suppress the activation as well as the repression of the target genes of the mating-type transcription factors, allowing the expression of the *MAT1-1* and *MAT1-2* target genes at a basal level. According to this model, the simultaneous expression of both classes of genes in a Δ*MAT* strain allow the development of the perithecia, but the low expression of these genes prevents the full development of the fruiting bodies.

The role of mating-type genes in *S. macrospora* has been recently reviewed (Pöggeler, 2007). The deletion of *Smta-1* does not prevent the formation of ascogonia and protoperithecia, but the mutant strain is unable to produce fruiting bodies and ascospores upon selfing (Pöggeler et al., 2006b). The Δ*Smta-1* strain is able to outcross, but the authors did not analyze the progeny, precluding any further comparison with the *G. zeae* system, particularly in relation to shifting to self-incompatible reproduction (see "Shifting from Self-Compatible to Self-Incompatible" below). Transcriptomic profiling of the Δ*Smta-1* strain by use of *N. crassa* cDNA microarrays has identified 107 genes that are significantly up- or downregulated in the mutant strain, but which of these genes are critical for mating has not yet been determined (Pöggeler et al., 2006b). Knockout mutants of the *S. macrospora* mating-type genes *SmtA-1*, *SmtA-2*, and *SmtA-3* have been recently reported (Klix and Pöggeler, 2008). SmtA-1 does not seem to be an essential regulator of the sexual development, as *SmtA-1* knockout mutants showed reduced fertility but were still able to produce sexual ascospores. Knockout mutants of *SmtA-3* showed no change in phenotype. The knockout mutant of *SmtA-2* was sterile and could no longer complete the sexual cycle. This phenotype is similar to that of *P. anserina SMR1* mutants. This is the first report showing possible mating-type gene function conservation between self-compatible and self-incompatible species.

Self-Compatible Eurotiomycetes

Analysis of the function of *MAT1* and *MAT2* in *A. nidulans* has been reported by two groups and has led to different conclusions.

The data of Paoletti et al. suggest that *MAT1* and *MAT2* do not control fertilization (Paoletti et al., 2007). Deletion of the *A. nidulans MAT1* gene does not prevent the formation of Hülle cells and cleistothecia under conditions

inducing the sexual cycle, but cleistothecia were fewer and smaller than those of control strains. Critically, cleistothecia were barren, with only a granular amorphous mass visible within, suggesting that the *MAT1* gene is primarily required for later stages of sexual development. A similar result was obtained with the deletion of the *MAT2* gene. Paoletti et al. have reported that transcription of the intact *MAT1* gene is strongly decreased if the *MAT2* gene is deleted (and vice versa) (Paoletti et al., 2007). This intriguing observation indicates that there is cross talk between the *MAT1* and *MAT2* genes and may suggest that single mutants are unable to outcross, even though they contain an intact mating-type gene. Overexpression of *MAT1* or *MAT2* genes has no effect on the expression of *MAT2* and *MAT1*, respectively, indicating that their cross talk is likely to involve other partners. Also, the overexpression of *MAT1* or *MAT2* does not induce the sexual cycle, but the overexpression of both genes results in growth arrest and formation of fertile cleistothecia, even on media that does not normally support sexual development. This experiment was performed with overexpressed genes in the same nucleus. Determining the behavior of a heterokaryotic individual with overexpressed *MAT1* and *MAT2* genes in different nuclei may help further the understanding of cross talk between mating-type genes.

The results of Miller's group give a somewhat different picture of the functions of *MAT1* and *MAT2* (Miller et al., 2005; Pyrzak et al., 2008). *MAT1* deletion strains produce fruiting bodies that stop their development at meiosis, after which the internal tissue eventually disintegrates and the cleistothecia lyse. The *MAT2* deletion strains produce only Hülle cells and protocleistothecia. According to this report, the *MAT2* gene would be required at least for fertilization, whereas the *MAT1* gene would operate later, during the development of the fruiting body.

Shifting Reproductive Modes: Are Self-Compatible Pezizomycotina Functionally Self-Incompatible?

Shifting reproductive modes provide new insights into mating-type gene expression, particularly the possibility that self-compatible mating types may function as self-incompatible ones. This suggestion was first proposed by R. L. Metzenberg and N. L. Glass (Metzenberg and Glass, 1990). They assumed that genetically self-compatible nuclei may become functionally self-incompatible, namely, that an epigenetic mechanism inactivates the *MAT1-1* or the *MAT1-2* functions in "self-compatible" *MAT1-1* and *MAT1-2* nuclei, as proposed by Claudio Scazzocchio for *A. nidulans* (Scazzocchio, 2006). This model will thereafter be referred to as the epigenetic self-incompatibility model. Shifting reproductive modes should also be beneficial on a practical level. Shifting a self-compatible species to self-incompatibility may facilitate the genetic analysis of important traits and the production of recombinant double-mutant strains from single mutants. Shifting a self-incompatible species to a self-compatible mode of reproduction is expected to ease the recovery of recessive mutations that only produce a phenotype during crosses. Two approaches have been studied to shift a self-incompatible species to self-compatibility: (i) the incorporation of both mating-type idiomorphs within a haploid genome and (ii) the expression of the mating-type from a closely related self-compatible species. The conversion of a self-compatible species to self-incompatibility was obtained by deleting the *MAT1-1* or *MAT1-2* counterparts, resulting in two compatible strains that have retained *MAT1-2* or *MAT1-1*, respectively. Alternatively, a strain with a complete deletion of the self-compatible mating type may be transformed with the idiomorphs from a closely related self-incompatible species.

Shifting from Self-Incompatible to Self-Compatible

The behavior of transgenic strains carrying both a resident and an opposite transgenic idiomorph has been reported in *P. anserina* (Picard et al., 1991) and *C. heterostrophus* (Turgeon et al., 1993). Crossing and selfing experiments with *P. anserina* and *C. heterostrophus* transgenic strains have shown that whenever the resident idiomorph of a transgenic strain is required to function in an outcross, the result is normal high fertility. In contrast, whenever the transgenic idiomorph is required to function in an outcross or a self, the efficiency of fertilization is normal, whereas ascospore production is low. This phenomenon has been termed interference (Wirsel et al., 1996). Ectopically integrated idiomorphs confer full fertility to strains that have a deletion of the resident idiomorph (Coppin et al., 1993; Wirsel et al., 1996), indicating that the expression of the ectopic idiomorphs is not deficient. However, this observation does not exclude that the fertility defect of the transgenic idiomorph results from an imbalance in the expression of transgenic and resident idiomorphs. To reduce this putative imbalanced expression, a *mat+* idiomorph has been integrated downstream from the *mat−* idiomorph in *P. anserina*. When this strain is selfed, fertility is low, but outcrossing with *mat+* and *mat−* tester strains shows reduced fertility in both crosses, suggesting that this structure affects the expression of both *mat+* and *mat−* functions (E. Coppin, personal communication). These approaches failed to reconstitute a fully fertile self-compatible strain. Taken together, these results indicate that self-compatibility necessitates more adjustments than the simple incorporation of two opposite idiomorphs in a haploid genome.

The expression of self-compatible mating-types in a self-incompatible species was first described in *C. heterostrophus* (Yun et al., 1999). The sterile *C. heterostrophus* MAT deletion strain was transformed with the *C. luttrellii MAT1-1-1*::*MAT1-2-1* gene. Abundant pseudothecia were formed when the transformants were selfed, but most of these pseudothecia showed lower fertility than those of the wild type (1 to 10% of wild-type ascospore production). A similar experiment was carried out with the *C. homomorphus MAT1-2-1*::*MAT1-1-1* gene (reviewed by Turgeon and Debuchy, 2007). In this experiment, the *C. heterostrophus* transformants produced barren pseudothecia. The fertility defect observed in these two experiments can be attributed to the interference phenomenon between the α1 and HMG domain of the fused transcription factors from self-compatible species. One possible way to test the impact of interference on fertility is to inactivate the α1 or the HMG domain in the fused protein. These mutations are expected to suppress interference and to increase fertility in transgenic *C. heterostrophus* containing the mutated fused gene. Alternatively, the fertility defect may result from a weak efficiency of the fused MAT regulatory protein in the activation of *C. heterostrophus* target genes. In that case, inactivation of the α1 or the HMG domain is not expected to increase fertility of transgenic *C. heterostrophus*. Interference between the two functions would argue for a mechanism suppressing that interference in *C. luttrellii* and *C. homomorphus*.

Shifting from Self-Compatible to Self-Incompatible

Successful conversion to a self-incompatible reproductive lifestyle by inactivating either the *MAT1-1* or *MAT1-2*

counterparts has been reported in G. *zeae* and provides intriguing insight into the functions of mating-type genes in this species (Lee et al., 2003). The Δ*MAT1-1*;*MAT1-2* and the *MAT1-1*;Δ*MAT1-2* strains are able to cross successfully with each other and with a self-compatible tester strain. Surprisingly, progeny analysis in the latter cross demonstrated a preferential association of the *MAT1-1*;*MAT1-2* wild-type nucleus with the nucleus containing a partial *MAT* deletion. This preferential recognition might be attributed to the undefined mechanism that favors mating of nuclei with different genetic backgrounds, a phenomenon known as "relative heterothallism" (see "Mating Systems" above). This explanation is unlikely here, however, as the strains used for crossing are isogenic, except for the partial *MAT* deletion and the genetic markers used in the cross. The preferential recognition of a self-compatible nucleus with a self-incompatible one appears to be driven by mating-type genes, which may function according to the epigenetic self-incompatibility model. The nucleus with a partial *MAT* deletion may induce the wild-type nucleus to inactivate one of its mating types, resulting preferentially in a compatible nucleus. Alternatively, *MAT1-1*;*MAT1-2* nuclei may undergo a random inactivation of *MAT1-1* or *MAT1-2*, and nuclei with a partial deletion of the *MAT* locus may recognize their compatible partners with a high efficiency, leaving their competitors originating from *MAT1-1*;*MAT1-2* without partners. The epigenetic self-incompatibility model may also explain the fertility decrease in Δ*MAT1-1*;*MAT1-2* × *MAT1-1*;Δ*MAT1-2* crosses. These crosses result in fewer perithecia than wild-type self crosses (10 to 20% of wild-type cross), and fertility of these perithecia was about 10% that of wild-type. If the putative epigenetic inactivating mechanism operates independently in each nucleus, many nuclei will be left with no functional *MAT* genes and will not participate in the formation of a progeny, thus explaining the fertility decrease.

Lu and Turgeon have expressed self-incompatible mating types in *C. luttrellii*. The deletion of the mating-type genes in *C. luttrellii* renders the strain completely sterile, proving that the fused *MAT1-1-1*::*MAT1-2-1* genes (Fig. 10) have conserved a function essential for sexual development (S. W. Lu and B. G. Turgeon, unpublished data [quoted by Turgeon and Debuchy, 2007]). The *C. luttrellii* strain with *MAT* deleted has been transformed, separately, with the *MAT1-1-1* or *MAT1-2-1* idiomorphs from self-incompatible *C. heterostrophus*. When these strains were crossed, it was found that a *C. luttrellii* transgenic strain carrying *Chet*MAT1-1-1 and a *C. luttrellii* transgenic strain carrying *Chet*MAT1-2-1 were able to mate in a self-incompatible manner and the fertility of the cross was similar to that of a wild-type *C. luttrellii* self. Unexpectedly, each transgenic strain of *C. luttrellii* is also able to self and to produce asci and full tetrads, but the number of asci is about 5 to 10% that of wild-type asci. This intriguing observation indicates that the target genes of the fused *C. luttrellii* mating-type transcription factor have features very different from those expected in self-incompatible species. In the latter group, the target genes of the mating-type transcription factors are believed to constitute two classes, the *MAT1-1* target genes activated specifically by the α1 protein and the *MAT1-2* target genes activated by the HMG protein. The findings reported by Lu and Turgeon suggest that *C. luttrellii* contains a single class of mating-type target genes that can be activated by the α1 and the HMG protein, i.e., the target genes have lost their *MAT1-1* or *MAT1-2* specificity. The epigenetic self-incompatibility hypothesis implies that a self-compatible species is in fact equivalent to a self-incompatible one, possessing two different classes of mating-type target genes, the first activated by *MAT1-1* genes and the second activated by *MAT1-2* genes. The behavior of transgenic *C. luttrellii* argues against the extension of the epigenetic self-incompatibility model to Dothideomycetes.

FERTILIZATION AND PHEROMONE/RECEPTOR SYSTEMS

Fertilization in several self-incompatible species has been described in detail. In most cases, the male cell attracts the trichogyne. Spermatia and microconidia do not generally respond to the trichogyne approach by undergoing morphological change, but recently Kuo et al. reported that macroconidia and arthroconidia of *N. crassa* produce a specialized conidial sex tube in response to the presence of the trichogyne of the opposite mating type (Kuo et al., 2007). There are several studies showing that pheromone and pheromone receptors are essential for the fusion of the trichogyne with the male cell in self-incompatible filamentous ascomycetes. In rare cases, trichogynes are nonfunctional and the fertilizing nucleus is thought to come from the mycelium. This has been reported for *Lachnea melaloma* and *Humaria granulata* (Chadefaud, 1960b), but these species may be self-compatible. How fertilization occurs in self-compatible fungi remains unclear. Pheromone and receptor orthologs needed in fertilization in self-incompatible fungi seem to be dispensable for selfing in self-compatible Pezizomycotina, but significantly some of these elements are required for developmental events after fertilization.

Pheromone/Receptor Systems and Self-Incompatible Species

There are several studies providing evidence that pheromone and pheromone receptors are essential for the fusion of the trichogyne with the male cell in self-incompatible members of the Pezizomycotina. Pheromone precursor genes have been isolated in the self-incompatible organisms *C. parasitica* (Zhang et al., 1993, 1998; Turina et al., 2003), *Magnaporthe grisea* (Shen et al., 1999), *N. crassa* (Bobrowicz et al., 2002; Kim et al., 2002; Kim and Borkovich, 2006), and *P. anserina* (Coppin et al., 2005b). Two types of pheromone genes were identified. One is related to the *MFα1* and *MFα2* genes of *S. cerevisiae* and will be referred to as the α-type. The *MFα1* and *MFα2* genes encode a precursor, which when processed produces multiple copies of a mature hydrophilic pheromone (reviewed by Davey et al., 1998). The α-type pheromone genes are expressed specifically in *MAT1-1* strains. However, these strains also contain the other pheromone gene type, which is related to *MFA1* and *MFA2* of *S. cerevisiae*, and will be referred to as a-type genes. The corresponding mature pheromones have a peptide backbone with a C-terminal residue that is both carboxymethylated and *S*-farnesylated (reviewed by Davey et al., 1998). They are expressed specifically in *MAT1-2* strains, even though these strains also contain the α-type genes. In addition to the mating-type control, the expression of the pheromone genes is tightly regulated according to the developmental stage of the mycelium. In *N. crassa*, the transcription of pheromone genes increases significantly under growth conditions that promote sexual development (Bobrowicz et al., 2002). In *P. anserina*, pheromone genes are transcribed at a very low level in growing mycelia, but they rank among the most transcribed genes when the mycelium reaches the stationary phase and becomes competent for sexual reproduction (J. Aitbenkhali, F. Bidard, and

S. Imbeaud, unpublished results). The presence of a conserved region just upstream from the initiation codon is an intriguing feature of a-type pheromone genes in filamentous fungi (Coppin et al., 2005b). It may suggest that translation and transcription both play a determinant role in the expression of pheromone genes. Deletion of the pheromone genes resulted in male sterility in the appropriate mating-type context. Further examination of the phenotype of these mutants in *N. crassa* showed that they were unable to attract the trichogyne of female organs of opposite mating type (Kim and Borkovich, 2006). Some reports proposed that pheromones may have another function besides fertilization, but to date there is no proof to support this hypothesis. The vegetative phenotypes of the repeat-induced point mutation mutant of the *mfa-1* gene in *N. crassa* (Kim et al., 2002) were subsequently attributed to mutations outside the *mfa-1* gene (Kim and Borkovich, 2006).

C. parasitica contains two genes encoding an a-type pheromone (Zhang et al., 1993, 1998) and one gene encoding an α-type pheromone (Turina et al., 2003). Deletion of the gene encoding the α-type pheromone results in the expected male sterility. Not surprisingly, the deletion of one copy of the two genes encoding the a-type pheromone did not prevent fertilization, but this deletion resulted in barren perithecia and defects in asexual sporulation. However, N. Van Alfen's group subsequently reported that the deletion encompasses several small open reading frames (ORFs), only one of which corresponds to the pheromone (Turina et al., 2003). They proposed that these small ORFs might be involved in asexual sporulation and in a developmental phase after fertilization.

Fertile crosses were obtained in *P. anserina* between strains deleted for all pheromone precursor genes, indicating that the pheromones do not play an essential function after fertilization (Coppin et al., 2005b). However, it was not verified whether ascogenous hyphal development was impaired in the fusion between the basal and lateral uninucleate cells (Fig. 2A). This step might rely on pheromone/receptor intercellular recognition (Egel, 1992), and a defect during this event may not be detected. Indeed, if the number of ascogenous hyphae is high enough, many asci may be produced independently of the uninucleate cell fusion ending the crozier formation. The decrease in the number of asci may then be barely detectable. It is thus necessary to check if croziers are formed to reach a final conclusion.

Pheromones are expressed in a mating-type-dependent manner, raising the question of the behavior of strains coexpressing a deregulated pheromone gene and its cognate receptor. These questions have been addressed in *N. crassa* and *P. anserina*. In *N. crassa*, the deregulated α-type pheromone gene (*ccg-4*) was introduced into a *MAT1-2* (*mat a*) strain (Kim and Borkovich, 2006). Conidia produced by this strain can attract the trichogyne of female organs from *mat a* tester strains, but cell fusion seems to fail. It is likely that cell fusion requires pheromones as well as other cell-type-specific products. The strain expressing *ccg-4* in a *mat a* context has been further tested for self-fertilization. It displayed larger and darker protoperithecia, some of which became as large as perithecia observed in wild-type crosses. However, the structures lacked defining features of perithecia, suggesting a block prior to the onset of meiosis. It is likely that additional determinants encoded by the *mat A* mating type are necessary to complete the sexual cycle. For *P. anserina*, a similar experiment was performed with deregulated copies encoding the a-type and the α-type pheromone in a *mat*− and *mat*+ context, respectively (Coppin et al., 2005b). In contrast to *N. crassa*, no induction of protoperithecial development was observed, indicating that the *mat*+ and *mat*− mating types repress a posttranscriptional processing of the α-type and a-type pheromone precursor, respectively. In agreement with this conclusion, mutations in the mating-type genes led to efficient self-fertilization, indicating that the repression of posttranscriptional processing is relieved. The self-fertilized perithecia produced a progeny, but this was also a feature of individuals in which mating-type genes are mutated (Arnaise et al., 2001a). This indicates that this fertility cannot be attributed unambiguously to the presence of the deregulated pheromone gene.

The functions of the *pre-1* gene, encoding the predicted receptor for the a-type pheromone, and *pre-2*, encoding the predicted receptor for the α-type pheromone, have been analyzed in *N. crassa* (Pöggeler and Kück, 2001; Kim and Borkovich, 2004, 2006). The deletion of *pre-1* resulted in female sterility in a *mat A* strain. Expression analysis indicated that *pre-1* and *pre-2* are transcribed in *mat A* and *mat a* strains, respectively. Unexpectedly, *pre-1* transcripts were detected in a *mat a* strain, but transcript levels were more than 100-fold lower than those in the *mat A* strain (Kim and Borkovich, 2004). Similarly, low but detectable levels of *pre-2* transcripts were observed in *mat A* strains (Kim and Borkovich, 2006). This observation led to speculation about possible transcriptional regulation of pheromone genes by a transduction cascade involving the receptors. In agreement with this hypothesis, the loss of the PRE-1 pheromone receptor strongly reduced the transcription of the α- and a-type pheromone genes in *A* and *a* strains, respectively (Kim and Borkovich, 2004). Reciprocally, the loss of the PRE-1 cognate pheromone decreased the transcription of the *pre-1* gene, but the loss of the PRE-2 cognate pheromone did not affect the transcription of the *pre-2* gene (Kim and Borkovich, 2006). These results suggest that there is a complex regulating pathway between pheromones and receptor, but further investigations are needed to understand this circuit.

Pheromone/Receptor Systems and Self-Compatible Species

Three self-compatible fungi, *S. macrospora*, *A. nidulans*, and *G. zeae*, have been inspected for the function of pheromones and receptors in fertilization. Two pheromone precursor genes and two pheromone receptor genes were identified in *S. macrospora* (Mayrhofer et al., 2006). Single knockouts of either one of these genes did not result in any defect in the sexual cycle. If the pheromone/receptor systems function as in self-incompatible species, the loss of both pheromones (or both receptors) and the loss of the pheromone and receptor genes under the control of the same mating-type factors should lead to impaired fertilization. Surprisingly, all these double mutants in *S. macrospora* displayed various degrees of fruiting-body maturation. In the double receptor knockout, protoperithecia became pigmented but remained small, and ascus development was completely prevented. The loss of the *MAT1-1* pheromone and receptor genes resulted in the formation of wild-type-sized perithecia up to 6% of wild-type level. These perithecia produced a few ascospores that were not viable. A similar result has been obtained with a strain containing a knockout of the *MAT1-2* pheromone and receptor genes. The double pheromone mutant was the least impaired strain, as wild-type-sized perithecia production reached 37% that of wild-type. Cytological analyses indicated that 20% of these perithecia contained ascus rosettes

similar to those in the wild type. A yeast assay performed by Mayrhofer and Pöggeler provided evidence that the α-type pheromone and its predicted receptor interact and trigger a pheromone response, indicating that they are functional (Mayrhofer and Poggeler, 2005). Moreover, the yeast assay indicated that the α-type pheromone is secreted by *S. macrospora* in the culture medium; however, *S. macrospora* produces no male elements to fertilize ascogonia. These data indicate that pheromone/receptor systems are not required for fertilization in *S. macrospora* but are required at a later stage of development. It would be interesting to test if the pheromone/receptor systems are involved in the synthesis of sexual morphogens that promote the development of the fruiting body.

In *A. nidulans*, two G-protein-coupled receptors (GPCRs), similar to the pheromone-sensing receptors of *S. cerevisiae*, have been identified (Dyer et al., 2003). Strains with knockouts of these genes produced fewer and smaller cleistothecia than those of the wild type and contained a reduced number (5% of the wild type) of ascospores that were viable (Seo et al., 2004), suggesting a postfertilization role for the receptors. The double mutant was unable to produce cleistothecia upon self-fertilization, equivalent to the *S. macrospora* double mutant (Mayrhofer et al., 2006). Surprisingly, outcrosses between two double-mutant strains resulted in wild-type levels of cleistothecia and viable ascospores. It is therefore possible that among the nine GPCRs identified in the *A. nidulans* genome (Han et al., 2004), the two pheromone receptors most similar to those of *S. cerevisiae* are necessary for self-fertilization, while one of the seven other GPCRs may serve for outcrossing. The best candidates for this function may belong to a group of three putative GPCRs that appear to be unique in filamentous fungi and that share high levels of similarity within the group. The members of this group are GprC, GprD, and GprE. GprD coordinates hyphal growth and sexual development. However, the deletion of *gprC* and *gprE* appears to cause no detectable phenotype, but the authors do not specify if they tested for possible function during outcrossing (Han et al., 2004).

Two pheromone precursor genes (*ppg1* and *ppg2*) and two pheromone receptor genes (*pre1* and *pre2*) were identified in the *G. zeae* genome (Kim et al., 2008; Lee et al., 2008). The *ppg1* and the *pre2* genes encode the α-type pheromone and its putative cognate receptor, respectively. Fertility tests with single knockout mutants for either one of these genes revealed a 50% decrease in the number of fertile perithecia, but the total number of perithecia is similar to that of the wild type (Kim et al., 2008; Lee et al., 2008). The presence of immature perithecia suggests that this pheromone/receptor system has a nonconventional function after fertilization. In contrast, knockout mutants of the a-type pheromone-encoding gene (*ppg2*) and its cognate receptor-encoding gene (*pre1*) were not different from the wild type in perithecial formation (Kim et al., 2008; Lee et al., 2008). Thus, these genes may be nonfunctional vestiges of the recent evolutionarily change from a self-incompatible to a self-compatible lifestyle. The quadruple mutant showed a tendency to produce more immature perithecia, similar to that of the *ppg1* and *pre2* mutants (Kim et al., 2008). In addition to their function after fertilization, *ppg1* and *pre2* play conventional roles in chemoattraction of female tissues by male cells (Lee et al., 2008). For instance, deletion of *ppg1* in the male strain dramatically decreased the effectiveness of spermatia for fertilizing a female strain impaired in self-fertilization by a knockout of *ppg1*.

Pheromone and Receptor Genes in Asexual Filamentous Ascomycetes

Putative orthologs of the α-type pheromone and pheromone receptor genes have been identified in the genome of *A. niger* (Pel et al., 2007) and *A. oryzea* (Galagan et al., 2005). The a-like pheromone genes are either absent or, more probably, difficult to locate, as they encode proteins of 20 to 30 residues with little conservation.

MAKING THE FRUITING BODY

The elaboration of a functional fructification depends on several environmental factors, as discussed in "Initiation of Sexual Reproduction" above. It can thus proceed only if a set of steps has occurred normally, including proper reserve material scavenging, specific physiological triggers, and good environmental conditions. It is thus not surprising that only a few ascomycete species have been tamed enough to produce fructifications regularly and efficiently in laboratory conditions. Moreover, it is also not unexpected, in those species that do, that several of the mutants affected in various processes ranging from amino acid biosynthesis and translation to respiration and cell structural component are unable to produce fruiting bodies (see Pöggeler et al., 2006a, for a table that lists some of the affected genes). The fruiting bodies are formed by two essentially different tissues: the hymenium resulting from fertilization, in which the sexual process per se occurs, and the sterile envelope that originates from the maternal parent and is likely to have a protective role. Assays screening for mutants have been carried out to decipher the process in *P. anserina* (Simonet and Zickler, 1972, 1978) and in *A. nidulans* (Swart et al., 2001). Additional sterile mutants recovered in many genetic screens have also been studied in *N. crassa* (reviewed by Raju, 1980).

Differentiation of the Dikaryotic Hyphae

After fertilization, dikaryotic hyphae emerge from the multinucleate dikaryotic cell and divide according to a tightly regulated developmental program to form crozier cells, as described above in "Hymenium Organization and Ascospore Production." The upper dikaryotic cell of the crozier will differentiate into asci, while the basal and lateral cells will fuse and form a new crozier and so on. Several cytological observations have been performed with a wide variety of ascomycetes to decipher the various steps of this conserved developmental process (Dangeard, 1894; Benjamin, 1955; Raju, 1980; Sautter and Hock, 1982; Zickler et al., 1995). However, very little is known about the genes and mechanisms that control these differentiation events. To our knowledge, only five altered phenotypes for the formation and/or differentiation of the dikaryotic hyphae have been described (Raju and Newmeyer, 1977; Simonet and Zickler, 1972, 1978; DeLange and Griffiths, 1980; Zickler et al., 1995). Among the corresponding mutants, some have been obtained spontaneously (*N. crassa Ban* mutants [Raju and Newmeyer, 1977]) or through UV or nitrosoguanidine mutagenesis (*N. crassa prf* mutant [Raju, 1987]; *P. anserina pex2* [previously *car1*] and *cro1* mutants [Berteaux-Lecellier et al., 1995, 1998]), and others have resulted from targeted mutations (*P. anserina mat* [Zickler et al., 1995] and *pex10* and *pex12* mutants [Peraza-Reyes et al., 2008]).

Crozier Formation

All crosses involving *P. anserina mat* mutants lead to selfish nuclei that can migrate alone in the ascogenous hyphae and form uninucleate rather than binucleate croziers, if

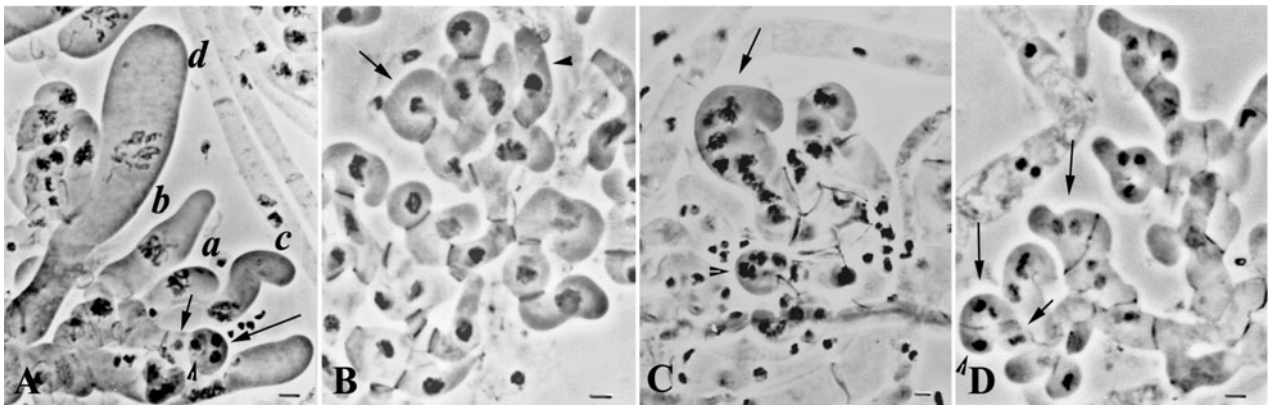


FIGURE 11 Crozier formation in *P. anserina* mutant strains. (A) Wild-type-like ascus development. Dikaryotic hyphae emerge from a plurinucleate cell and form, after one or two divisions, the crozier cells in which the two nuclei divide. This coordinate mitosis gives rise, after septum formation, to three cells: an upper binucleated cell (long arrow) and two uninucleated cells, one lateral (arrowhead) and one basal (arrow). Karyogamy will take place in the upper cells, which contain two nuclei of opposite mating types. Nuclear fusion is followed by meiosis and elongation of this upper cell, now called an ascus (*a* through *d*). A young small ascus where karyogamy has just occurred (*a*) and a midprophase ascus (*d*) are visible. (B) In a cross between a *mat* mutant and the wild type, uninucleated croziers are formed (arrow); the arrowhead points to an enlarged cell that might evolve into an ascus. (C) Crozier phenotype of a *cro1* × *cro1* mutant cross. Most croziers are plurinucleated and often form giant croziers (arrow), in which nuclei divide synchronously, but no septa are formed. On the left of this large crozier is a smaller one (arrowhead), still containing an abnormally high number of nuclei but exhibiting a normal size (when compared with the wild-type crozier size in A). (D) Binucleated croziers issued from a *pex2* × *pex2* mutant cross. Differentiation of the dikaryotic cells is similar to that in the wild type; however, instead of undergoing karyogamy, the two nuclei isolated in the upper binucleated cell (long arrows) divide again to form another crozier cell. Lateral and basal cells are indicated by an arrowhead and an arrow, respectively. Scale bar, 5 μm. Light micrographs are a kind gift from D. Zickler (IGM; Université Paris Sud-11, France); rosettes of asci are stained by hematoxylin (see Zickler et al., 1995, for details).

produced from a homozygous wild-type cross (Fig. 11; compare A and B, long arrow). Surprisingly, these uninucleate croziers can undergo haploid meiosis, but the ascospores produced usually abort (Zickler et al., 1995). Importantly, the fact that ascospore formation occurs in crosses involving *mat* mutants suggests that the corresponding *mat* genes are dispensable at a late stage of reproduction.

Conversely, the *N. crassa Ban* (Raju and Newmeyer, 1977) and *Prf* (Raju, 1987) mutants and the *P. anserina cro1-1* mutant (Simonet and Zickler, 1978) form multinucleate croziers. The *cwl-1* and *cwl-2* mutants of *N. crassa* also exhibit plurinucleate croziers. However, these are not of interest here, as they result from an inability to form septa, even in mycelia, and thus do not result from defects in crozier establishment per se (Raju, 1992). *N. crassa Ban* and *Prf* mutant croziers are very similar to those of the *P. anserina cro1* mutant. However, in the *P. anserina cro1-1* mutant, nearly all crozier cells are plurinucleate. Conversely, in *N. crassa Ban* and *Prf* mutants, only croziers that are formed late become multinucleate. *N. crassa* mutants have particular features compared with *cro-1* mutants: *Ban* and *Prf* are dominant and female-sterile, and each ascus essentially produces one giant ascospore. Interestingly, *Ban* and most *Prf* multinucleate croziers remain haploid (their nuclei never undergo karyogamy), whereas karyogamy, meiosis, and sporulation can proceed in *P. anserina cro1-1* multinucleate croziers (Berteaux-Lecellier et al., 1998). Together, the data obtained from *mat* and *cro1-1* mutant analyses show that, at least in *P. anserina*, meiosis and ascospore formation do not rely on the number of nuclei isolated in the crozier.

Unfortunately, only one of the mutants displaying multinucleate croziers has been further characterized. *P. anserina* CRO1 belongs to the conserved family of UCS proteins (Berteaux-Lecellier et al., 1998; Hutagalung et al., 2002). This class of proteins is assumed to interact with various myosins, and recent studies involving yeast have shown that UCS proteins contribute to their stability and motor function (Lord et al., 2008). Even if UCS protein mutants exhibit various phenotypes, the altered processes are related to myosin-based processes (reviewed by Yu and Bernstein, 2003). The UCS protein Rng3p in *S. pombe* contributes to the formation and maintenance of the cytokinetic contractile ring, notably via its interaction with the myosin-II motor protein (Lord et al., 2008). Such a role of CRO1 may explain the *P. anserina cro1-1* mutant phenotype, in which actin belts and septa are often misplaced or absent during ascogenous hyphae formation. CRO1 may control the establishment and/or maintenance of a true cellular state through its interaction with myosins.

The Mitotic-Meiotic Shift

In *P. anserina* homozygous crosses involving *pex2*, *pex10*, or *pex12* mutants, a later step of ascogenous hyphal differentiation is altered. In fact, the establishment of dikaryotic crozier cells is similar to that of the wild type. However, the upper cell of the crozier never engages in meiosis; instead,

the two nuclei divide again to form another crozier cell and so on. The perithecia thus become filled with hundreds of croziers in which no diploid stage is observed (Fig. 11, compare D and A) (Berteaux-Lecellier et al., 1995; Peraza-Reyes et al., 2008). Even if this phenotype may be a sign of a defect in nuclear fusion, vegetative-karyogamy studies have shown that this process is not altered in *pex2* mutants and suggest that karyogamy per se is not affected (Berteaux-Lecellier et al., 1995). In filamentous ascomycetes, premeiotic replication occurs before karyogamy (Zickler, 2006). Therefore, the absence of nuclear fusion may only be the outcome of a deficiency in meiotic engagement. PEX2, PEX10, and PEX12 are peroxisomal membrane proteins and together form the RING-finger complex, which belongs to the peroxisomal matrix protein import machinery (Platta and Erdmann, 2007). Peroxisomes are nearly ubiquitous organelles involved in several metabolic pathways among which some are ubiquitous and others are more specific regarding the organisms or cell types (van den Bosch et al., 1992; Reumann, 2000; Boisnard et al., 2004). In humans, alterations in peroxisomes lead to severe diseases (Wanders and Waterham, 2005). Also, even though their roles in various developmental programs have become increasingly demonstrated (Titorenko and Rachubinski, 2004; Terlecky and Titorenko, 2009; see also chapter 15), the link between the pleiotropic phenotype of the peroxisomal diseases in humans and the peroxisomal deficiency remains unclear. The overall studies performed with *P. anserina* peroxisomal mutants suggest that the role of the RING-finger complex in meiotic engagement does not rely on the classical features of peroxisomal matrix protein import and should provide new insights on the fungal peroxisomal role in differentiation (Bonnet et al., 2006; Peraza-Reyes et al., 2008) (see chapter 16 for details).

Making the Envelope

Surprisingly, very few female-sterile mutants have been investigated for their implication in the building per se of the envelope of the fructification, and our knowledge of this stage is still rudimentary. This step is difficult to analyze, as one must disentangle three types of phenomena: (i) those that have occurred before reproduction and that impair fruiting-body formation, when the abnormal growth of the mycelium does not permit further sexual development; (ii) those that affect the dikaryon; and (iii) those that affect the envelope per se. To this end, heterothallic species are better suited than homothallic species, as fertilization can be controlled. Thus, a distinction can be made between the events that precede and those that follow fertilization. However, recovering recessive fertility mutants from heterothallics can be time-consuming, unlike in homothallics. In addition, developmental studies have centered mostly on ascohymenial ascomycetes, and this in fact relates almost exclusively to *A. nidulans* and a handful of sordariomycetes (mainly *N. crassa*, *S. macrospora*, and *P. anserina*), due to the ease with which their sexual reproduction is triggered on petri dishes. The following is thus based on studies conducted with a few species, and it is highly likely that ascolocular ascomycetes produce fructifications based on completely different processes.

In ascohymenial ascomycetes, development of the fructification proceeds after the differentiation of the female gametangium. Depending on the species, fructifications may partially form before fertilization, likely to protect the female gametangium (e.g., protoperithecia in some sordariomycetes). The ascogonium must send some signal(s) to the neighboring hyphae to permit the early development of the fructification. However, in the absence of fertilization, fructifications do not fully develop; moreover, the final form of the fructification is not reached, indicating that the dikaryon plays a crucial role in shaping the surrounding sterile tissues. Interestingly, *P. anserina* mutants of the *SMR1* gene, blocked at the transition between the dikaryon and the ascogenous hyphae, form small but normally shaped fructifications with necks (E. Coppin, personal communication). Mutants with defects in later stages of the sexual process, such as those affected in meiosis or ascospore maturation, form normal ascoma. Their centra reach a larger size, unlike those of *SMR1* mutants, even if they are constituted of paraphyses or croziers mostly. This indicates that a morphogenetic signal is sent by the dikaryon early during maturation to form a neck, and that a "growth" signal is subsequently sent to accommodate the growing centrum. To which extent the envelope exerts a reciprocal control on the formation of the dikaryon is mostly unknown. However, without a protective envelope, it is expected that sexual development may not proceed (see below). Mutants described as having normal-looking empty perithecia are found in several species (Esser and Graw, 1980; Tharreau et al., 1997). Therefore, at least in some species, it is possible to dissociate the formation of the envelope and dikaryon signaling, suggesting that the dikaryon activates a program, which then acts autonomously. The relevant signaling pathway(s) emanating from the dikaryon is unknown. Also, the interplay between external factors (light, gravity, oxygen tension, etc.) and internal signals (morphogens) to produce a fructification with a definite shape remains unknown. However, part of the pathway involved in building the fructification is described below.

The various steps of development can be analyzed with the help of mosaics, as has previously been done in animal embryogenesis. The first reported study used a mosaic between a wild-type strain and a cell-autonomous pigment-deficient mutant of *N. crassa* that otherwise displayed wild-type sexual reproduction (Johnson, 1976). This analysis has allowed the elucidation, in part, of the origins of the various constituents of the fructification, e.g., the inner, median, and outer layers of the envelope, as well as the neck and the ascus rosette. Since then, mosaics between color and development mutants have been used in *P. anserina* to determine the role of genes during protoperithecium and perithecium development. The color mutant used is *psk1-193*, which is mutated in the polyketide synthase acting in the first step of melanin synthesis (Coppin and Silar, 2007). This mutant is fertile and produces pigment-less fruiting bodies. Pigment production is cell autonomous, as fully pigmented, fully unpigmented, and mosaics are obtained in a cross between *pks1-193* and the wild type. The first developmental mutants analyzed are impaired in fruiting-body formation but are fully able to differentiate ascogonium and spermatia. The genes affected in these mutants encode an NADPH oxidase (PaNox1 [Malagnac et al., 2004]), a pezizomycotina-specific protein of unknown activity (IDC1 [Jamet-Vierny et al., 2007]), and three kinases, members of an MAPK module (PaASK1, PaMKK1, and PaMpk1 [Kicka and Silar, 2004; Kicka et al., 2006]). *pks1-193/PaNox1*$^-$ and *pks1-193/IDC1*$^-$ heterokaryons of opposite mating types produce nonmelanized fruiting bodies only, whereas those constructed with *pks1-193* and the *MAPK* module mutants produced nonmelanized, fully melanized, and mosaic fructifications. Thus, it was deduced that PaNox1 and IDC1 are required in the perithecium and cannot diffuse into it, whereas the MAPK module either diffuses into, or is dispensable in, the

ascoma. Green fluorescent protein-tagging of PaMpk1 showed that it is expressed in the mycelium surrounding the fructification but seemingly not in the perithecium, suggesting that the MAPK module does not diffuse into the fruiting body but rather acts in the surrounding mycelium. These results strongly suggest that the neighboring hyphae actively participate in the making of fruiting bodies, possibly through the mobilization of nutrients to fuel its development, adding another layer of signaling. In *A. nidulans*, a mutanase involved in glucan degradation was shown to be dispensable for cleistothecium development (Wei et al., 2001), suggesting that nutrient supply may be complex.

In *P. anserina*, additional heterokaryons were made with a Δ*mat* strain and developmental mutants. Δ*mat* lacks the mating-type region and is rarely able to engage in fertilization, but it may be able to provide sterile hyphae to construct the fructification. Δ*mat*/*PaNox1*$^-$ *mat*$^+$/*PaNox1*$^-$ *mat*− heterokaryons produce several wild-type-like ascoma that produce 100% *PaNox1*$^-$ ascospores. Thus, PaNox1, although necessary in the perithecium, is not required in fertile tissue, and hence, its role is restricted to the building of the fructification, i.e., the envelope. The same is true of IDC1. Interestingly, these data also show that without an envelope, the hymenium does not develop. Additionally, overexpression of IDC1 produced enlarged protoperithecia in the absence of fertilization (Jamet-Vierny et al., 2007). Among these, few mature into fertile fructifications after fertilization. A continuous supply of nutrients partially suppressed this defect, suggesting that a premature exhaustion of the mycelium reserves may be involved, thus providing another link between the state of the mycelium and an ability to complete sexual reproduction.

The present model to account for perithecium development derived from *P. anserina* is summarized in Fig. 12. It emphasizes the role of NADPH oxidase in signaling between the fructification and the mycelium and thus the

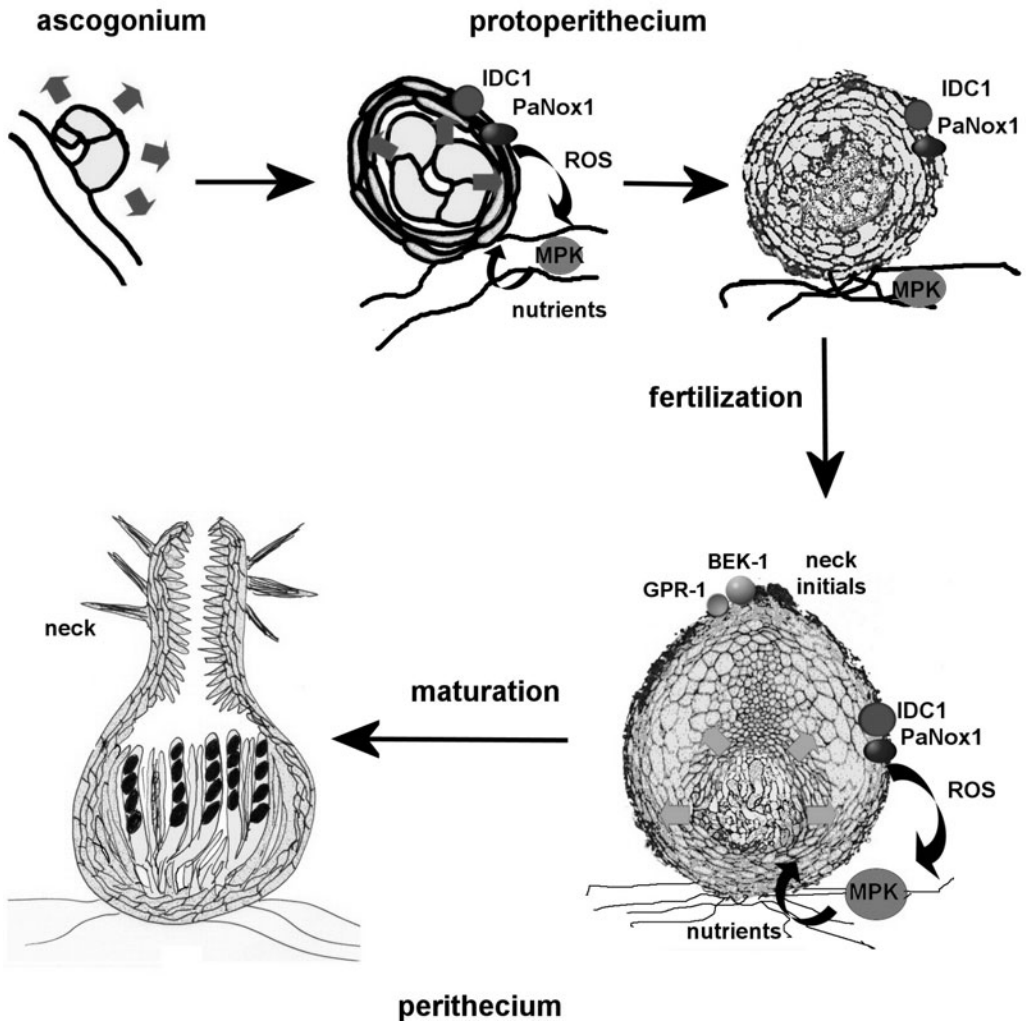


FIGURE 12 Cross talk between mycelium and perithecium envelope. The model is based on data obtained with *P. anserina* and *N. crassa*. After differentiation, the ascogonium sends a signal to neighboring hyphae to form a protective envelope. The signaling pathway containing PaNox1, IDC1, and the PaMpk1 module is necessary to mobilize these hyphae. Then, in the absence of fertilization, development stops. After fertilization, the same signaling pathway is necessary for the development of the fructification. PaNox1 and IDC1 act in the wall of the fructification, whereas the MAPK module acts in the surrounding hyphae. Signaling then results in the transfer of nutrients to the developing fructification. At later stages of development, a neck is formed under the control of the BEK-1 transcription factor and GPR-1 receptor.

role of redox signaling in differentiation, as previously suggested (Hansberg and Aguirre, 1990; Aguirre et al., 2005). Reactive oxygen species may have multiple roles in development, as thioredoxin genes have been shown to be important for proper fructification in *P. anserina* (Malagnac et al., 2007). Analysis of mosaics has indicated that, unlike PaNox1, these proteins are necessary in fertile tissue (Malagnac et al., 2007). Thioredoxin has multiple roles and may be involved in signal transduction through the oxidation of their cysteines. A role of redox signaling was also reported in *A. nidulans*, as an NADPH oxidase (Lara-Ortiz et al., 2003) and a thioredoxin (Thon et al., 2007) also control fertility in this species. Thus, redox signaling may be widely conserved in Ascomycetes to produce ascomata. It would be interesting to test whether the NADPH oxidase and thioredoxin act in *A. nidulans* at the same level as in *P. anserina*. There is a genetic system available in this species for mosaic analysis: previously, the *blAI* color mutant was successfully used to ascertain the origin of nuclei in the cleistothecium wall in *A. nidulans* (Zonneveld, 1988). Additionally, the mosaic of this mutant and a development mutant affected in the *rcoA* gene, which encodes a transcription repressor related to *S. cerevisiae* TUP1, has shown that *rcoA* expression is necessary in the cleistothecial wall (Todd et al., 2006). The *N. crassa* ortholog, *rco-1*, is necessary for the differentiation of protoperithecia (Yamashiro et al., 1996). It would be very interesting to check whether *rco-1* expression is required in the perithecium envelope in Sordariomycetes. In *C. heterostrophus*, a mosaic system based on an *albino* mutant should also further understanding in ascolocular development (Lev et al., 1999). Similarly, double-mutant analysis with developmental mutants should now be conducted to reconstruct the regulatory pathways operating during development, in both Ascohymeniales and Ascoloculares.

The downstream targets of the signaling pathways are unclear. It is obvious that hyphae constructing the fructification behave differently from those that permit vegetative growth. It is not yet clear at which step of the cell physiology these changes are triggered and which components are involved. However, the endoplasmic reticulum seems to be of major importance for sexual reproduction in *S. macrospora* (Nowrousian et al., 2007), and it was suggested that it plays a major role in wall remodeling (Busch and Braus, 2007). This remodeling of hyphae is especially apparent during neck formation in Sordariomycetes (Fig. 12). During this process, the perithecium envelope changes at a defined position. Cells lining the outer and inner sides of the canal differentiate. Those on the outside may accumulate pigments or transform into ornamentations in some species. An ostiole permitting ascospore release is usually created during neck maturation. This complicated process in *N. crassa* is controlled by the GPCR GPR-1 (Krystofova and Borkovich, 2006). It requires G proteins and the BEK-1 homeodomain transcription factor (Krystofova and Borkovich, 2006). An absence of light in *P. anserina* results in neckless perithecia full of ascospores (Berteaux-Lecellier, unpublished), indicating that light is a major morphogen involved in defining the axis of the fructification.

Ascosporogenesis and Ascospore Biology

Ascospore differentiation has yet to be investigated at the molecular level, but detailed cytological studies have been performed (Raju and Perkins, 1994; Thompson-Coffe and Zickler, 1994; Thompson-Coffe et al., 1999). Once fully mature, ascospores are usually expelled from the fructification to ensure dispersal. F. Trail and H. E. Hallen reported that in perithecium-forming fungi the asci fire singly in succession, suggesting the presence of a regulatory mechanism that coordinates discharge (Hallen and Trail, 2008). Although some data concerning the biomechanics of the discharge process are available, very little information concerning the molecular aspect of the process is known, and the coordinating mechanism is even more elusive (reviewed by Trail, 2007). Several studies have pointed to the importance of potassium, chloride, and calcium ion channels in the discharge process. In *G. zeae*, the genes encoding these ion channels were found to be constitutively expressed or showed increased transcript accumulation during ascus formation (Hallen et al., 2007). The deletion of the gene encoding the L-type calcium ion channel Cch1 in *G. zeae* abolishes ascus discharge, even after a 3-week incubation of the fruiting bodies (Hallen and Trail, 2008). Transcriptomic analysis of the mutant revealed that deletion of the *Cch1* gene alters transcription of 1,428 genes at the time of ascospore production, obscuring the relation between the observed phenotype and the function of the protein. However, the addition of calcium to the culture medium can suppress the ascus discharge defect, suggesting that Cch1 directly affects cell components to cause ascus propulsion rather than by influencing transcription. In *G. zeae*, the four genes encoding the potassium transport protein are expressed during ascospore formation, particularly *fg03834*, for which transcription is twofold greater during this period (Hallen et al., 2007). In *P. anserina*, the deletion of two genes encoding potassium transport proteins did not affect the ascus discharge mechanism (Lalucque and Silar, 2004), indicating that not all ion transport proteins are involved in this process.

The final step of the sexual process is the germination of ascospores to yield the growing vegetative mycelium. Germination is relatively varied, depending on the fungal species. Some ascospores require dormancy, whereas others germinate immediately. Some require defined conditions (temperature, nutrient, pH, etc.) to trigger germination, whereas others will germinate in water. Finally, some germinate through one or a few germ pore(s), whereas others germinate from any region of the ascospore. In mutants that have a poor ascospore germination rate, it is difficult to know if germination is impaired because of maturation defects of the spore or because of abnormality in the signaling required for germination. In *P. anserina*, ascospores germinate through a germ pore without dormancy on a defined medium that contains ammonium acetate. Melanin is required for medium-regulated germination (Malagnac et al., 2004; Coppin and Silar, 2007), as nonmelanized ascospores germinate on any medium including water agar. The NADPH oxidase PaNox2 (Malagnac et al., 2004) and the PaPls1 tetraspanin (Lambou et al., 2008) are required for the germination of melanized ascospores, but not for nonmelanized ones. By contrast, the Mgpls1 tetraspanin, which is orthologous to PaPls1, is not required for *M. grisea* nonmelanized ascospore germination. These data suggest that melanization is required for the proper regulation of germination in *P. anserina* and that PaNox2 and PaPls1 play a role in regulating germination, but not in germination per se. In *N. crassa*, the MAK-2 MAP kinase module mutants (Kothe and Free, 1998; Pandey et al., 2004; Li et al., 2005) are impaired in ascospore germination. Deletion of the orthologous *PaMpk2* in *P. anserina* also resulted in melanized ascospore germination impairment (F. Malagnac and P. Silar, unpublished data). However, an absence of melanin

restores a normal rate of germination in the *PaMpk2* mutants, as similarly observed for *PaNox2* and *PaPls1*. Thus, at least in *P. anserina*, failure to germinate does not result from a structural defect affecting the gross anatomy of the ascospore but rather from a regulatory defect or a structural defect restricted to the germ pore.

SUMMARY

Recent data have begun to shed light on the various molecular processes occurring during sexual reproduction in filamentous Ascomycetes. In addition to the well-known variety of body plan exhibited by Pezizomycotina fruiting bodies, sequencing has uncovered a large set of mating-type structures, all based on a common pattern. The remarkable fluidity of the mating systems raises major questions: what are the functions of the mating-type genes, and is there a common functional scheme throughout the various mating types? Although sequencing of mating types provides valuable information on mating-type structure and evolution, only genetic analysis allows a better understanding of the roles that mating-type genes have during sexual development. The genetic tools necessary to study sexual reproduction, such as developmental mutants, inactivated genes, constructed mosaics, and double mutants, are available for several species, thus covering the diverse range of organisms observed in Pezizomycotina. A concerted approach similar to that already adopted in animal developmental studies should now increase the comprehension of sexual reproduction in fungi.

Major questions regarding the function of mating-types genes and the development of the fructifications remain unanswered. Indeed, it is now well established that *MAT1-1-1* and *MAT1-1-2* genes control fertilization by using pheromone/receptor genes in self-incompatible Pezizomycotina, but little is known about their roles after fertilization. The few indications that come from *P. anserina*, *N. crassa*, and *C. heterostrophus* indicate that they control the formation of biparental ascogenous hyphae and meiosis. However, the mechanisms under the control of the mating-type transcription factors remain unknown, and the function of *MAT1-1-2* is still unknown, although it does appear to be an essential fertility gene in Sordariomycetes. The functions of mating types in self-compatible pezizomycotina are even less known than those in self-incompatible fungi. An important issue at stake is to determine if the dual mating types of self-compatible species are functionally similar to those of self-incompatible fungi, as proposed by Metzenberg and Glass (Metzenberg and Glass, 1990), possibly by using an epigenetic system silencing either one of the dual mating types. Future studies should be directed at identifying the target genes of the mating-type transcription factors and analyzing the function of these genes by large-scale approaches, ranging from cellular biology to biochemistry and genetics. These genes must be involved in the building of the fructifications, which clearly requires communication between the fertile tissue, the envelope, and the surrounding mycelium. The careful analysis of developmental mutants already available and analysis at various stages of development will provide some answers. New genetic screens should help to provide new tools to fill the gaps in our knowledge. Finally, comparisons between the various models should provide some clues about the evolution of sexual development in fungi, an aspect that should not be dissociated from evolution and the function of mating-type genes, as this pluridisciplinary approach has proved so fruitful and exciting in animals and plants under the denomination of "evo-devo."

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Mating and Sexual Morphogenesis in Basidiomycete Fungi

LORNA CASSELTON AND MICHAEL FELDBRÜGGE

The mating-type genes of fungi have provided remarkable insights into the ways in which genes determine cell fate, how cells communicate, and how cell fusion triggers major changes in gene expression. Just a few genes are involved, and these map to specialized regions of the genome, known as the mating-type (*MAT*) loci, a single locus in ascomycete fungi (see chapter 33) and either one or two loci in basidiomycetes.

Ascomycetes and Basidiomycetes are classed as members of the Dikarya. A characteristic feature of this group of fungi is the long delay that occurs between fertilization (cell fusion) and nuclear fusion. Following fertilization, the nuclei from each mate divide several times to form what in many species is a mycelium containing two nuclei per cellular compartment; hence, the name dikaryon. In ascomycetes, the dikaryotic cells are generally referred to as ascogenous hyphae, because they develop within the confines of the fruiting body and give rise to the numerous ascus cells in which nuclear fusion, meiosis, and sporulation occur. In basidiomycetes, the dikaryon is a free-living mycelium, and this is the predominant form in nature. It gives rise to fruiting structures composed almost entirely of dikaryotic cells when nutrient, light, and temperature conditions are favorable. The mating pathways of filamentous ascomycete and basidiomycete fungi are clearly similar, relying on pheromones and cognate receptors for cell communication as well as specific transcription factors to regulate gene expression. Yet surprisingly, the genes at the *MAT* loci appear to be unrelated. Fungal *MAT* genes encode critical transcription factors, but in basidiomycetes, unlike ascomycetes, they also encode the pheromones and receptors. The reason for this difference between the two major divisions of the Dikarya will become evident when we consider specific aspects of the basidiomycete lifestyle.

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HISTORICAL PERSPECTIVE

Historically, heterothallism in Basidiomycetes was first described in the hymenomycetes *Coprinopsis cinerea* (*Coprinus fimetarius*/*C. lagopus*/*C. macrorhizus*/*C. cinereus*) and *Schizophyllum commune* by the Portuguese mycologist Mathilde Bensaude in 1918 and the German mycologist Hans Kniep between 1915 and 1918 (Kniep, 1928), respectively. Kniep went on to show that four mating types segregated from a single fruiting body, consistent with the presence of two unlinked mating-type loci, which he termed the "*A* and *B* mating type factors." In order to form the fertile dikaryon, mating partners needed to have different alleles of both factors. Kniep also recognized that both mating-type factors were multiallelic and that many different mating types existed in the population. These observations were confirmed for *C. cinerea* later by scientists such as Hanna in 1925 and Brunswick in 1926. An excellent account of these historical discoveries has been given by John Raper (Raper, 1966).

Quintanilha and Pinto-Lopes estimated that some 65% of hymenomycete species were like *C. cinerea* and *S. commune* in having two unlinked mating-type loci (Quintanilha and Pinto-Lopes, 1950). These species are said to be tetrapolar because four mating types segregate at meiosis. Some 25% have just a single mating-type locus and are termed bipolar (two mating types segregate), and the remaining 10% are self-fertile. *C. cinerea* and *S. commune* have been the major models for hymenomycete studies, providing details of the molecular basis of mating-type determination. This knowledge has enabled studies with nonmodel species that show how different hymenomycete mating systems are related. It is now more appropriate to refer to the *A* and *B* "factors" as *MATA* and *MATB*.

At present, by far the best-understood basidiomycete model with respect to mating is *Ustilago maydis*. This heterobasidiomycete is a member of the Ustilaginales, fungi that cause smut diseases of grasses. Smuts are plant pathogens, and mating is critically required for pathogenicity. The dikaryon of *U. maydis* is an obligate pathogen of its host plant, whereas the asexual stage is yeast-like and nonpathogenic.

Heterothallism in a related species, *Microbotryum violaceum* (*U. violacea*), was recognized as early as 1919 by Kniep (Kniep, 1928). *M. violaceum* is a bipolar species with just two mating types, but *U. maydis*, as described by Hanna in 1929, is tetrapolar. Soon after, in 1927, Christensen provided evidence that *U. maydis* had multiple mating types, like the hymenomycetes, but it was not until 1952 that Rowell and DeVay demonstrated that there were only two alleles at one *MAT* locus (which they designated *a*) but many more at the other locus (designated *b*), now thought to have 25 alleles (Rowell and DeVay, 1954). Excellent accounts of early studies on *U. maydis* are given by Banuett (Banuett, 1995, 2007) (see chapter 39).

Another important model basidiomycete species is the dimorphic heterobasidiomycete *Cryptococcus neoformans*, a human pathogen that infects the central nervous system causing meningoencephalitis in immunocompromised patients (see chapter 44) (Hull and Heitman, 2002). *C. neoformans*, like *U. violaceum*, is bipolar, with just two alleles in the population. Of particular interest, the dikaryon of *C. neoformans* is not pathogenic, but the haploid yeast-like cells of just one of its two mating types are pathogenic (*Mat*α) (Kwon-Chung and Bennett, 1978; McClelland et al., 2004).

REPRESENTATIVE LIFE CYCLES

Since mating is intimately linked to the life cycle in the Basidiomycetes, we chose the life cycles of two model species, the filamentous *C. cinerea* (Kües, 2000) and the dimorphic and pathogenic *U. maydis* (Banuett, 1995), to illustrate key steps in mating and sexual development (Fig. 1A and B, respectively). In *C. cinerea*, the haploid sexual spores (basidiospores) are produced on the undersurface of the mushroom fruiting body and germinate to form a filamentous mycelium, which is generally termed a monokaryon, because the cells are predominantly uninucleate. The monokaryon is sexually sterile but produces abundant uninucleate asexual spores (oidia) (Fig. 1A). Cell fusion in hymenomycetes is mating-type independent. A compatible mating is sensed only after cell fusion when compatible nuclei with different mating types are present in the same cell. The result is the formation of a vigorous dikaryotic mycelium that is readily distinguished from the monokaryon by structures known as clamp connections that form each time the tip cell divides. Subsequent to hyphal fusion there is a reciprocal exchange of nuclei, following which the donor nuclei migrate through the existing mycelial cells of the recipient to establish binucleate tip cells. Once both nuclei (dikaryotic pair) are present in the tip cell, this triggers a complex division. A clamp cell forms midway along the length of the tip cell, and one nucleus enters into this cell. Both nuclei divide in synchrony, and new cell walls are laid down, cutting off one daughter nucleus in the clamp cell and the other in the subterminal cell. The clamp cell grows backwards, and a short peg produced by the subterminal cell grows to meet it (Badalyan et al., 2004; Buller, 1931). The cells fuse to form the clamp connection, and the clamp nucleus is released into the subterminal cell (Fig. 1A). This complex sequence of events is repeated each time the dikaryotic tip cell divides and is particularly relevant when we come to consider the functions of the mating type genes.

Given the right environmental stimuli, the dikaryon differentiates the complex mushroom fruiting bodies, in which the dikaryotic nuclear pair finally fuses in specialized basidial cells that cover the undersurface of the cap. The diploid nuclei undergo meiosis almost immediately, and the four haploid nuclei produced enter the basidiospores attached on the outside of the basidial cell. There are some 10 million basidia on the undersurface of a typical mushroom, and due to the fact that the fruiting body deliquesces rapidly after maturation, meioses are remarkably synchronized (Fig. 1A) (Pukkila, 1994) (see chapter 7).

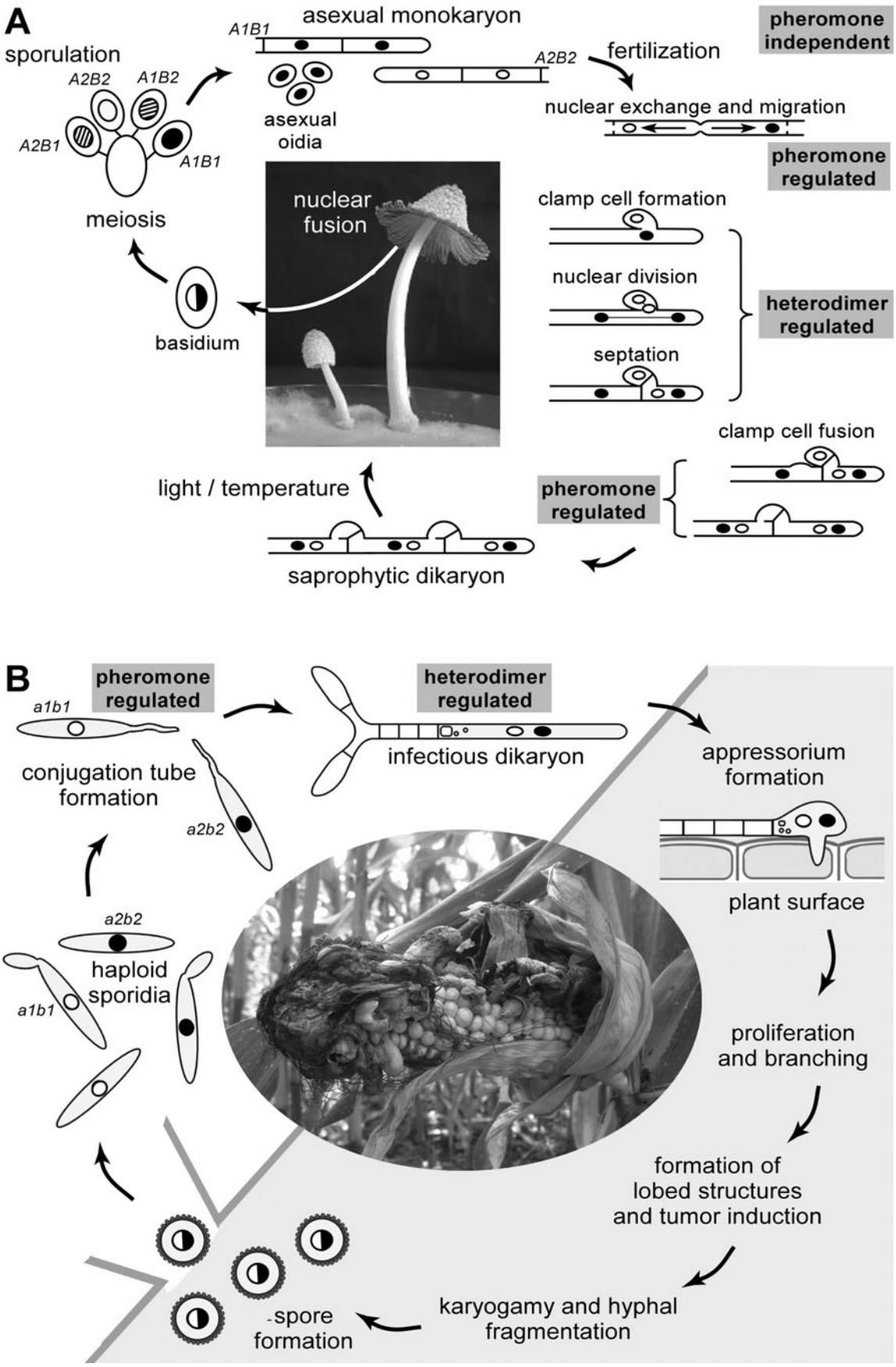
Swiezynski and Day generated incompatible fusions between monokaryons with the same *A* or the same *B* mating-type alleles (common *A* and common *B* heterokaryons) and determined that different *B* genes, which we now know to encode pheromone and receptor genes, were required for nuclear migration and clamp cell fusion (Swiezynski and Day, 1960). However, different *A* genes were shown to be necessary for tip cell division and formation of the clamp cell. In *S. commune*, similar experiments confirmed the roles of the *A* and *B* genes but also showed that nuclear migration was active in common *A* heterokaryons. These common *A* heterokaryons of *S. commune* have an unmistakable phenotype that is termed "flat" (Raper, 1966).

As its name implies, *U. maydis* is a pathogen of *Zea mays*. Infection results in the formation of tumors on different organs of the host, and these are particularly dramatic in the kernels (Fig. 1B). Tumors are filled with black diploid teliospores, hence the name smut. On germination, the teliospore gives rise to a short filament within which meiosis occurs, generating four haploid cells that bud off haploid sporidia. Sporidia are yeast-like cells that divide by budding and are saprophytic and nonpathogenic. Unlike *C. cinerea* and other hymenomycetes, cell fusion is *MAT* dependent. Mating requires pheromone attraction between compatible cells; both cells secrete pheromones and in response to pheromone binding to appropriate receptors, produce long filamentous conjugation tubes that fuse at their tips. A few hours later, a new filament emerges from the fusion point, and this is dikaryotic. The dikaryon, as mentioned earlier, is an obligate pathogen of the host, and by means of a specialized infection structure designated the appressorium, it pierces the plant cuticle to enable colonization of the internal plant tissues (Fig. 1B). Since mating is required to form the infectious dikaryon, the *MAT* genes also control pathogenicity, thus explaining the intense interest that has been focused on this species (Feldbrügge et al., 2006; Kahmann and Kämper, 2004).

In 1955, Rowell established the different roles that *a* and *b* genes have in mating (Rowell, 1955). Dikaryon formation requires that alleles of both *MATa* and *MATb* be different. Cells with the same *a* alleles but different *b* alleles fail to produce conjugation tubes and do not fuse, whereas cells with different *a* alleles but the same *b* alleles can fuse but form a weak filament that is nonpathogenic. The *a* genes, which we now know encode the sex pheromones and receptors, are thus implicated in cell fusion, and the *b* genes encode the main determinant for filamentous growth and pathogenicity (see below).

MOLECULAR CHARACTERIZATION OF THE *MAT* LOCI

The first fungal mating-type genes to be cloned and sequenced were those of the ascomycete *Saccharomyces cerevisiae* (Herskowitz, 1988). Understanding the role that these *MAT* genes play in cell-specific gene expression and mate attraction is particularly relevant to understanding mating in all other members of the Dikarya, even though the lifestyles of these fungi and the actual genes at the *MAT*


A
sporulation
A2B2
A1B2
A2B1
A1B1
meiosis
basidium
asexual monokaryon
A1B1
A2B2
asexual oidia
fertilization
pheromone independent
nuclear exchange and migration
pheromone regulated
nuclear fusion
clamp cell formation
nuclear division
septation
heterodimer regulated
clamp cell fusion
pheromone regulated
light / temperature
saprophytic dikaryon
B
pheromone regulated
a1b1
conjugation tube formation
a2b2
heterodimer regulated
infectious dikaryon
appressorium formation
plant surface
proliferation and branching
formation of lobed structures and tumor induction
karyogamy and hyphal fragmentation
spore formation
a2b2
haploid sporidia
a1b1

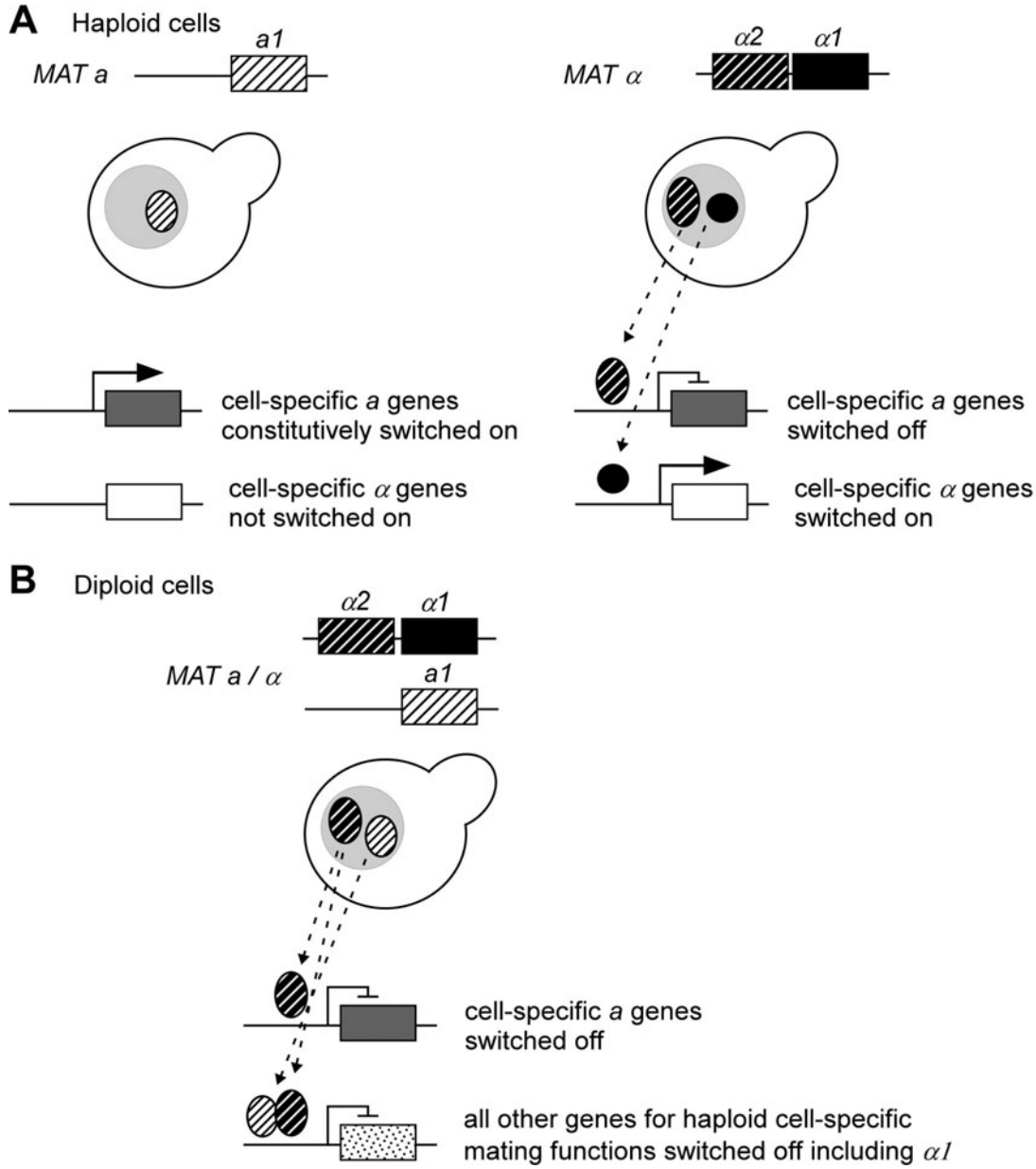


FIGURE 2 Organization and function of genes at the *S. cerevisiae MAT* locus. (A) The *MAT* locus in *MATa* cells encodes a single protein, a1, that plays no role in regulating cell-specific *a* genes. The idiomorphic *MAT* locus in *MATα* cells encodes two proteins, α1, which activates cell-specific α genes, and α2, which represses cell-specific *a* genes. (B) In diploid cells with both alleles of *MAT*, α2 represses cell-specific *a* genes, and a new transcription factor generated by heterodimerization between α2 and a1 proteins represses all genes required for haploid mating functions.

loci may differ. Before looking at the basidiomycete *MAT* loci, it is worth being reminded of what happens during mating in *S. cerevisiae*. Crucial to mating is cell signaling and recognition. Figure 2 summarizes the essential features of *MAT* locus structure and gene function in *S. cerevisiae*. The alternative versions of the *MAT* locus are known as **a** and **α**. Haploid cells secrete small peptide pheromones that act as chemoattractants. *MATa* and *MATα* cells secrete different pheromones, and each produces a *MAT*-specific receptor that spans the cell membrane and is a member of

FIGURE 1 Life cycles of the filamentous hymenomycete *C. cinerea* (A) and the dimorphic heterobasidiomycete *U. maydis* (B). Details are given in the text.

the G-protein-coupled receptor family. Receptors bind the pheromone secreted by cells of the other mating type. The pheromones are derived from precursor molecules by post-translational cleavage and modification. **a** pheromone is a lipopeptide that is C-terminally carboxymethylated and farnesylated. It is secreted via an ABC transporter (Ste6p) (Chen et al., 1997). In contrast, α pheromone precursor contains two to four copies of the peptide pheromone. These are cleaved and secreted via the classical Golgi system (Kurjan, 1993). The different maturation pathways of the pheromones and the different receptors that bind them (Ste2p and Ste3p) require that each haploid cell express a subset of genes not expressed in the other mating type. This is achieved by transcription factors that bring about this differential gene regulation in haploid cells.

MATa cells have just a single functional gene at *MAT* (*a1*) that encodes a protein not required in haploid cells; **a** cell-specific genes are expressed constitutively (Fig. 2). In *MATα* cells, these **a**-specific genes must be repressed. This is the role of the protein encoded by *MATα* gene *α2*. The *α1* gene is a transcriptional activator of *MATα* cell-specific genes.

Binding of pheromone to a compatible receptor causes the G protein bound to its inner surface to dissociate and to trigger an internal phosphorylation cascade involving a mitogen-activated protein kinase (MAPK) pathway. The target of this pathway is the transcription factor Ste12p, which binds upstream of genes necessary to enhance the pheromone signal and bring about cell fusion and karyogamy. Also phosphorylated by the MAPK is the Far1 protein that leads to cell cycle arrest in G_1, which prepares nuclei to fuse following karyogamy. Once cells have fused, the a1 and α2 proteins heterodimerize to form a new transcription factor that represses all genes required for pheromone signaling, including the *MATα1* gene. a1 and α2 are members of the homeodomain family of proteins, but they have distinctly different homeodomain sequences (Bürglin, 1994) and are distinguished by being referred to as *HD1* (*α2*) and *HD2* (*a1*) genes and proteins (Kües and Casselton, 1992).

Pheromone signaling and homeodomain protein dimerization also play an essential role during mating in basidiomycetes. As mentioned previously, one of the *MAT* loci in tetrapolar species encodes pheromone precursors and cognate receptors (Fig. 3). The other *MAT* locus encodes the two classes of homeodomain proteins corresponding to a1 and α2 in *S. cerevisiae* (see Fig. 5). Unlike *S. cerevisiae* and other ascomycetes, the *MAT* loci of basidiomycetes are not idiomorphic (i.e., encoding functionally different genes [see chapter 33]). Hence, each locus has the same genes, and compatible mating partners bring together different alleles.

U. maydis presents a paradigm for understanding the functions of the mating-type genes and downstream regulation. With an understanding of this relatively simple system it is easy to see how the complexity apparent in the corresponding hymenomycete loci evolved (see below).

PHEROMONES AND COGNATE RECEPTORS

The *MATa* locus of *U. maydis* was characterized on the molecular level by Bölker et al. (Bölker et al., 1992). Each of the two alleles of *MATa* contains one gene encoding a pheromone receptor and another encoding a pheromone precursor. There are just two alleles of *MATa*. *a1* contains the receptor gene *pra1* and the pheromone gene *mfa1*, whereas *a2* contains the receptor gene *pra2* and pheromone gene *mfa2* (Fig. 3A). The DNA sequences of these alternative versions of *MATa*, although not idiomorphic, are very different, ensuring that the receptor and pheromone genes cannot be recombined into compatible combinations. As in *S. cerevisiae*, the mature pheromones are secreted and act as chemoattractants. Binding of a1 pheromone to the Pra2 receptor and a2 pheromone to Pra1 activates the pheromone response in each cell, resulting in growth of elongated conjugation tubes (Fig. 1B).

In another smut, *Sporisorium reilianum*, there are three alleles of *MATa* (Schirawski et al., 2005), two of which have relatively similar sequences suggesting a recent duplication-diversification event (Fig. 3B). Interestingly, all three loci encode two pheromones. The *MATa2* locus in both species has acquired extra genes, *rga2* and *lga2*, neither of which is required for mating, but which are most likely involved in uniparental inheritance of mitochondria (see below) (Bortfeld et al., 2004). However, as we see below, the regulation of these genes is dependent on *MAT* gene function.

Hymenomycetes appear to be unique among fungi in having evolved large families of pheromones and receptors. Several versions of the *B* locus of *C. cinerea* have been sequenced, and allele *B42* is illustrated in Fig. 3C (Halsall et al., 2000; O'Shea et al., 1998; Riquelme et al., 2005). Other loci have the same organization but may contain even more pheromone genes (Casselton and Kües, 2007; Riquelme et al., 2005). The genes are multiallelic, and as a result of gene duplication each *B* locus takes its particular specificity from three closely linked subloci, each containing similar but functionally independent genes. As in the *a* locus of *U. maydis*, a unique DNA sequence ensures that genes within any version of a sublocus are maintained as a functional unit. None of the *B* genes present in a haploid genome can activate development. Mating partners are compatible if they bring together different alleles of just one of these three subloci. This is sufficient to give at least one compatible receptor-pheromone combination to promote pheromone signaling. The three subloci appear to be contiguous, but comparisons of several loci show that recombination has occurred during evolution so that different alleles of the three are brought together in different combinations. An analysis of 13 *B* loci identified two alleles of sublocus 1, five alleles of sublocus 2, and seven alleles of sublocus 3 (Riquelme et al., 2005), sufficient to generate 70 ($2 \times 5 \times 7$) unique cross-compatible *B* specificities, close to the 79 predicted by Day (Day, 1963).

Data from transformation experiments with single pheromone genes are consistent with genes in the three subloci being functionally independent in *C. cinerea*. However, since it was possible to test these interactions only in a background containing other pheromone and receptor genes, some intergroup activity could not be ruled out (Riquelme et al., 2005). Corresponding studies with *S. commune* (Fowler et al., 1999) are more definitive because these were carried out in a host strain in which the entire *B* gene complex was deleted (Raper and Raper, 1973).

In *S. commune*, the *B* locus has only two subloci, corresponding to the *Bα* and *Bβ* loci (Fowler et al., 2004; Vaillancourt et al., 1997; Wendland et al., 1995). The detailed organization of the *Bα3-Bβ2* complex is illustrated in Fig. 3D (Fowler et al., 2004). Classical studies identified nine alleles of *Bα* and nine alleles of *Bβ* (a predicted 18 different receptors). In *Bα3-Bβ2* there are three pheromone genes in the α sublocus and as many as eight in the *β* sublocus. Together, these 11 pheromones are sufficient to activate in a highly specific fashion all the nonself receptors found in the other 16 subloci. These data are summarized in

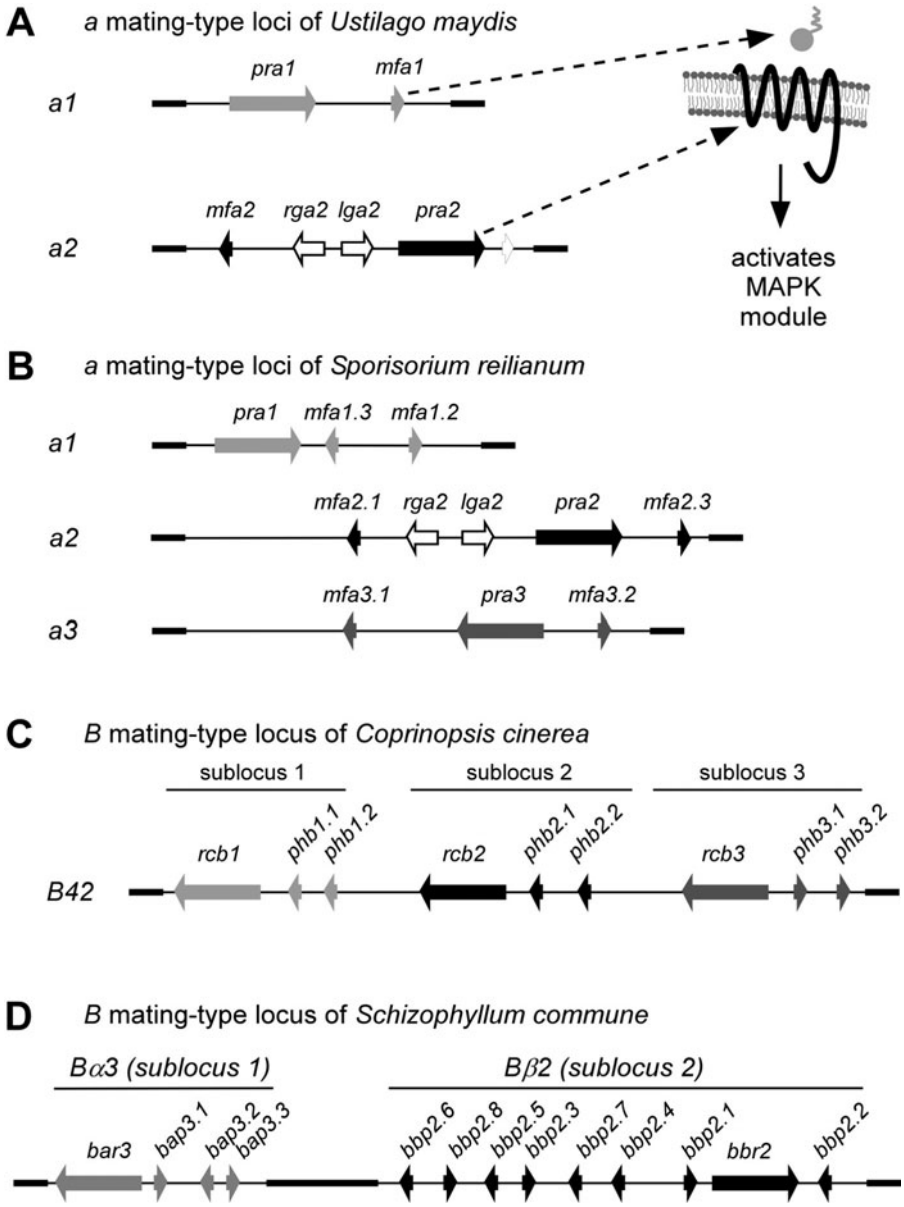


FIGURE 3 Organization of the basidiomycete *MAT* loci encoding pheromones and receptor genes: *U. maydis* (A); *S. reilianum* (B); *C. cinerea* (C); and *S. commune* (D). The receptor genes are designated *pra1* and *pra2* in *U. maydis*; *pra1*, *pra2*, and *pra3* in *S. reilianum*; *rcb1*, *rcb2*, and *rcb3* in *C. cinerea*; and *bar3* and *bbr2* in *S. commune*. The prefix *mfa* is used to denote the pheromone genes of *U. maydis* and *S. reilianum*, *phb* in *C. cinerea*, and *bap* or *bbp* in *S. commune*. Different fill motifs represent different alleles of genes in *U. maydis* and *S. reilianum* and paralogous genes in different subloci in *C. cinerea* and *S. commune*. *lga2* and *rga2* in the *U. maydis* and *S. reilianum a2* locus encode mitochondrial proteins, likely involved in uniparental inheritance. A compatible pheromone-receptor interaction that activates the pheromone response is shown for *U. maydis*.

Table 1. Perhaps most surprising was the discovery that two of the pheromones encoded at the *Bα* sublocus could activate one of the *Bβ* receptors and three of the pheromones encoded at the *Bβ* sublocus could activate *Bα* receptors, showing that the functional separation of α and *β* genes is incomplete. This discovery explained early findings in classical studies that certain *αβ* complexes could not be recombined even when they conferred different specificities (Koltin and Raper, 1967).

The pheromones encoded at the *MAT* loci of all basidiomycete fungi studied belong to the *S. cerevisiae* **a** lipopeptide pheromone family, and the receptors are correspondingly members of the *S. cerevisiae* Ste3p family (Vaillancourt and Raper, 1966). The mature pheromone is derived from a large precursor molecule that has a C-terminal CaaX box motif, a signal for C-terminal truncation, carboxymethylation, and farnesylation. So far, no secreted pheromones have been detected in hymenomycete fungi;

TABLE 1 Receptor specificities of the predicted 11 pheromones encoded at the *Bα3-Bβ2* locus of *S. commune*[a]

Pheromone[b]	Mature pheromone sequence	Pheromone receptor activated[c]												
Bap3 (1)	ERVGTGGTATAFC		α2	α4	α5									
Bap3 (2)	EKGGTSMAHAWC	α1	α2			α6								
Bap3 (3)	ERHGSGNMTYFC[d]			α4			α7						β8	
Bbp2 (6)	EREGDGNMTYFC[d]			α4			α7						β8	
Bbp2 (4)	DSPDGYFGGYC[d]							α8		β1				
Bbp2 (5)	DSPDGYFAGYC[d]							α8		β1				
Bbp2 (8)	DSPCGFGGGYC								β3					β9
Bbp2 (7)	DKPCGYGGGYC								β3					β9
Bbp2 (3)	DQITDYQSYC[d]							α9			β5			
Bbp2 (1)	EHGYGGSNVHGWC								β4			β6	β7	
Bbp2 (2)	EHEEDTDSNVHGWC								β4			β6	β7	

[a]Based on Fowler et al., 2004.

[b]Bap3 identifies the pheromones encoded at the Bα locus, and Bbp2 identifies the pheromones encoded at the Bβ locus. The numbers in parentheses indicate the number of pheromone species encoded at each locus.

[c]None of the pheromones activates the self receptors Bar3 and Bbr2.

[d]Pheromones that activate receptors in both α and β subloci.

however, likely sequences of mature peptides could be predicted using *S. cerevisiae* as a heterologous expression system (Olesnicky et al., 1999). The precursor molecules bear no obvious similarity to *S. cerevisiae* **a** pheromone precursor, but when expressed in yeast, they are processed to active pheromone that is secreted and activates a compatible basidiomycete receptor expressed on the surface of the host cell (Fowler et al., 1999; Olesnicky et al., 1999). By noting a conserved dipeptide sequence, most commonly glutamic acid (E) and arginine (R), N-terminal to the CaaX box in 12 sequenced precursors (Casselton and Olesnicky, 1998), a likely N-terminal processing site was predicted. Synthetic pheromones were designed and shown to activate the same receptor expressed in *S. cerevisiae* (Olesnicky et al., 1999). With large numbers of receptors and pheromones, only some of which can activate each other, it is of interest to detect how specificity might be determined. By predicting the mature peptide sequences of all characterized pheromones of *S. commune*, Fowler et al. (2004) were able to show that these fell into five distinct classes based on sequence differences. It was then possible to identify likely amino acids that were critical in determining pheromone-receptor specificity.

For *U. maydis*, the sequences of both pheromones a1 and a2 were obtained by purifying secreted peptides (Spellig et al., 1994). This allowed the generation of synthetic pheromones whose biological activity was dependent on the C-terminal hydrophobic tail (Koppitz et al., 1996). Mutational analysis of the a1 pheromone by alanine as well as D-amino acid scanning revealed that two central glycine residues as well as the C-terminal tryptophan are important for function (Szabó et al., 2002). Interestingly, the majority of pheromones from *S. commune* contain central glycines as well as a bulky aromatic amino acid near the C terminus (Table 1), indicating that this might be a common feature involved in receptor binding.

THE PHEROMONE SIGNALING NETWORK

Signal transduction processes during mating are initiated by the binding of pheromones to their cognate receptors. Thereby, specific signaling cascades that trigger defined cellular events such as increased pheromone secretion or cell cycle arrest are elicited (Fig. 4). A pheromone-activated MAPK module constitutes a core element of pheromone signaling that is widespread among ascomycetes as well as basidiomycetes. Such a module consists of three hierarchically organized kinases: a MAPK kinase kinase (MAPKKK) phosphorylates a MAPK kinase (MAPKK) that phosphorylates a MAPK. Important targets of MAPKs are transcription factors that are responsible for triggering the pheromone-regulated transcriptional response. However, pheromone signaling is not simply a linear pathway consisting of insulated signaling components but is connected to other pathways forming a complex and sophisticated signaling network that integrates environmental and pheromone signals (Davey, 1998; Elion, 2000; Feldbrügge et al., 2004; Idnurm et al., 2005).

In the Basidiomycetes, the best-studied example is pheromone signaling in *U. maydis*. Pheromone-stimulated sporidia secrete increasing amounts of pheromone, arrest the cell cycle, form conjugation tubes, and prepare for postfusion events (Fig. 4). This defined response is mainly regulated by a distinct transcriptional program (Feldbrügge et al., 2006; Garcia-Pedrajas et al., 2008; Kahmann and Schirawski, 2007). Genome-wide expression studies using DNA microarrays identified 375 pheromone-responsive

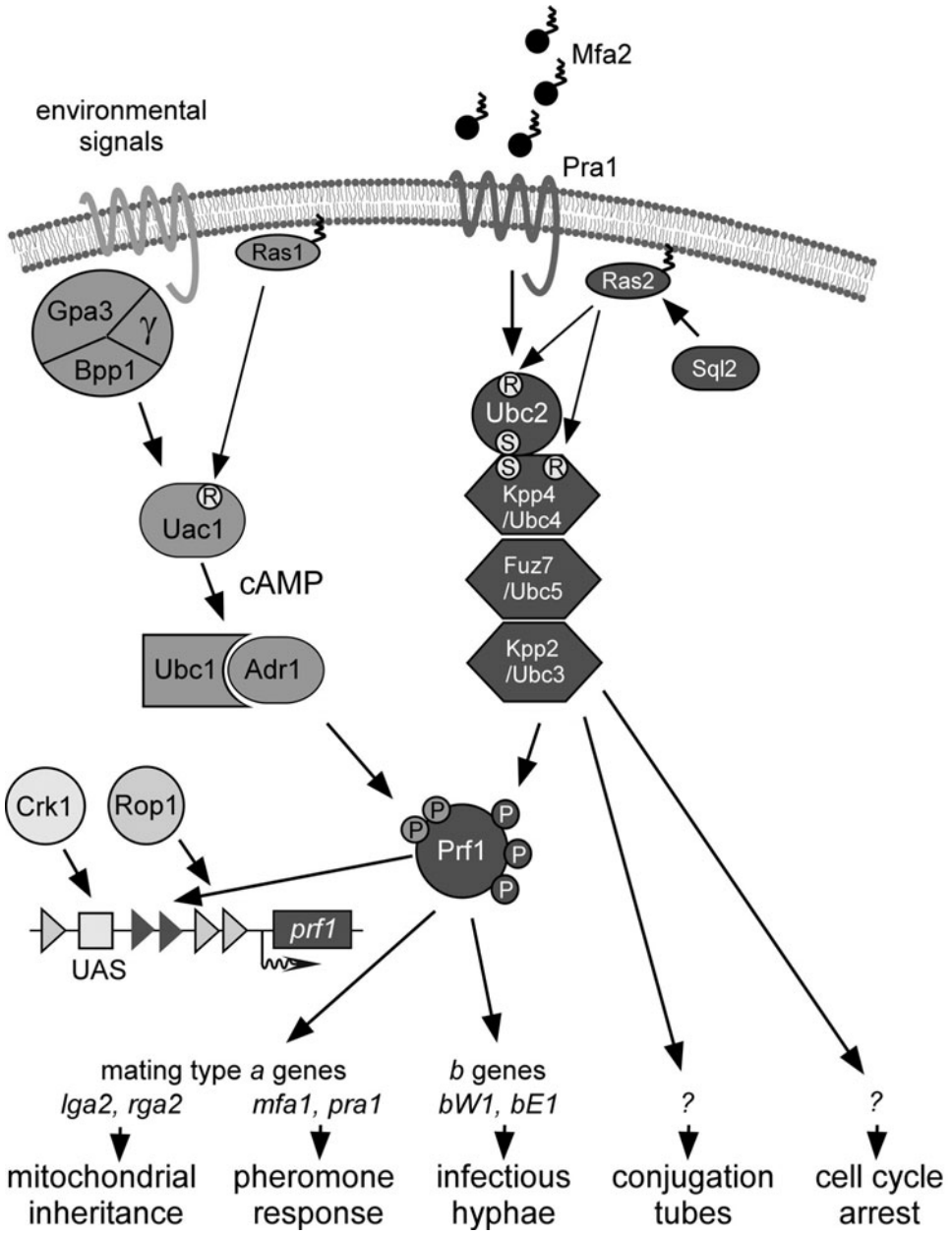


FIGURE 4 Pheromone signaling network during mating in *U. maydis*. Components of a conserved MAPK module (hexagons) communicate with a conserved cAMP signaling pathway (left). Phosphorylation of Prf1 through PKA and MAPK signaling (circled P) is used to differentiate between *a* and *b* gene expression. The novel MAPK Crk1 as well as HMG box transcription factors Rop1 and Prf1 regulated *prf1* expression transcriptionally (see the text for details) (figure modified from Feldbrügge et al., 2006).

genes. Bioinformatic analysis revealed that almost one-half of these genes encode novel factors without functional annotation. Among the induced genes with predicted functions were a number of genes that encode enzymes for N- and C-terminal pheromone processing as well as secretion (Zarnack et al., 2008). This is consistent with earlier results showing that expression of *mfa1/2* and *pra1/2* genes present at the *a* mating-type locus is pheromone induced (Urban et al., 1996). Thus, positive-feedback regulation of pheromone secretion and perception is important for recognition of the mating partner. Since comparable results were obtained in *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans*, this mode of stimulation constitutes a conserved feature of fungal pheromone signaling (Bennett et al., 2003; Roberts et al., 2000; Xue-Franzén et al., 2006).

In addition, expression of several transcription factor genes is induced upon pheromone perception, including *prf1*, *rbf1*, and both genes of the *b* mating-type locus (Hartmann et al., 1996; Urban et al., 1006; Zarnack et al., 2008). While Prf1 constitutes the key transcription factor during

pheromone signaling (Hartmann et al., 1996) (see below), the b heterodimer and the zinc finger transcription factor Rbf1 are particularly important for postfusion events (Scherer et al., 2006). Thus, pheromone signaling appears to trigger transcriptional cascades that guide and amplify cellular responses.

Another interesting pair of pheromone-induced genes is *lga2* and *rga2*, two additional genes present at the *a2* locus (Urban et al., 1996). These encode two mitochondrial proteins that have been implicated in determining uniparental mitochondrial inheritance (Bortfeld et al., 2004). It is likely that increased expression of these proteins is important to mark mitochondria of one mating partner. Thereby, mitochondria can still be recognized in a mixed cytoplasm after cell fusion in order to ensure uniparental inheritance.

Among pheromone-repressed genes with known function are various genes encoding cell cycle components such as B-type cyclin Clb1 (Garcia-Muse et al., 2004). This correlates with the pheromone-induced arrest of the cell cycle in G_2 (Garcia-Muse et al., 2003), preparing nuclei to divide in synchrony after cell fusion. This is in contrast to *S. cerevisiae*, where cell cycle arrest occurs in G_1.

The core element of pheromone signaling in *U. maydis* is a MAPK module that consists of MAPKKK Kpp4/Ubc4, MAPKK Fuz7/Ubc5, and MAPK Kpp2/Ubc3. Activation of this module elicits increased expression of mating-type genes, cell cycle arrest, and conjugation tube formation (Fig. 4) (Andrews et al., 2000; Banuett and Herskowitz, 1994; Mayorga and Gold, 1999; Müller et al., 1999, 2003b).

At present, it is unclear how the pheromone signal is transduced from the receptor to the MAPKKK Kpp4/Ubc4. A signaling component likely to be involved is Ubc2, since respective deletion strains are impaired in pheromone response and virulence. This putative adaptor protein shares similarity with the pheromone signaling component Ste50p from *S. cerevisiae*. It contains several protein interaction domains such as a sterile alpha motif (SAM), a Ras association domain (RA), and an Src homology domain (SH3) (Klosterman et al., 2008; Mayorga and Gold, 2001). A connection between Ubc2 and Kpp4/Ubc4 could be mediated by the SAM domains of both proteins (Fig. 4) (Klosterman et al., 2008; Müller et al., 2003b). Further candidates are the small G protein Ras2 and its potential activator, the guanine nucleotide exchange factor Sql2 (Müller et al., 2003a). Ras2 has the potential to feed into MAPK signaling via RA domains in Ubc2 and Kpp4/Ubc4 (Fig. 4) (Lee and Kronstad, 2002; Müller et al., 2003b).

Transcriptional profiling of strains harboring a genetically activated MAPK module revealed that MAPK signaling triggered a similar gene repression program as seen in response to pheromone. However, MAPK activation was not sufficient to trigger expression of numerous pheromone-induced genes, indicating that additional signaling pathways cooperate to elicit the full pheromone response. This demonstrated at the genome-wide level that networking is crucial for pheromone signaling (Zarnack et al., 2008).

Cross talk between MAPK signaling and a second signaling pathway during mating was already described in studies of heterotrimeric G proteins, which are coupled to receptors sensing extracellular signals. Initially, it was observed that the α subunit Gpa3 and the β subunit Bpp1 are important for mating (Müller et al., 2004; Regenfelder et al., 1997). However, mating defects could be rescued by addition of external cyclic AMP (cAMP) (Krüger et al., 1998). Further investigation revealed that both components are upstream of an evolutionarily conserved cAMP signaling pathway consisting of adenylate cyclase Uac1 (Gold et al., 1994) and cAMP-dependent protein kinase A (PKA). The latter is composed of the regulatory subunit Ubc1 and the catalytic subunit Adr1 (Fig. 4) (Dürrenberger et al., 1998; Garcia-Pedrajas et al., 2008; Gold et al., 1997). A molecular connection to mating was discovered by demonstrating that the intracellular cAMP level correlates with *mfa1* expression. Thus, low cAMP levels, e.g., present in *gpa3*Δ and *uac1*Δ strains, lead to reduced *mfa1* expression, causing defects in mating. Conversely, high cAMP levels result in elevated *mfa1* expression. Since an increase in *mfa1* expression could also be triggered by activation of the MAPK module, active PKA and MAPK signaling exhibit synergistic effects on expression of mating-type genes (Krüger et al., 1998).

Presently, it is unclear which receptor is coupled to the heterotrimeric G protein. Since mating is influenced by growth conditions, it has been speculated that favorable environmental conditions are sensed by the receptor of the PKA pathway. Therefore, sensing the presence of the plant could be coupled to mating. This might be particularly important for this plant pathogen, since later steps of its life cycle are strictly dependent on the host (Feldbrügge et al., 2004, 2006).

In order to understand the operation of complex signal transduction networks, it is mandatory to identify key nodes. For the pheromone response, this was achieved by demonstrating that the transcription factor Prf1 integrates PKA and MAPK signaling via phosphorylation by the terminal kinases Adr1 and Kpp2/Ubc3, respectively (Kaffarnik et al., 2003). Prf1 is essential for mating, since it confers basal as well as pheromone-responsive expression of the mating-type genes (Fig. 4) (Hartmann et al., 1996). The protein contains a high-mobility-group (HMG) box DNA-binding domain that specifically recognizes pheromone response elements (PREs) present in the regulatory regions of its target genes (Hartmann et al., 1996; Urban et al., 1996).

The activity of Prf1 is regulated at the transcriptional level as well as posttranslationally by PKA and MAPK phosphorylation (Fig. 4) (Kaffarnik et al., 2003; Müller et al., 1999). At the transcriptional level, *prf1* expression is induced via active MAPK signaling. This could be mediated at least in part by positive autoregulation employing two PREs present in the *prf1* promoter that are bound by Prf1 in vitro (Brefort et al., 2005; Hartmann et al., 1999). Moreover, the HMG box protein Rop1 (regulator of *prf1*) is essential for *prf1* expression in axenic culture, and its absence causes defects in mating. This transcription factor recognizes three specific promoter elements of the *prf1* promoter (Brefort et al., 2005). However, on the plant surface, *rop1*Δ strains express sufficient amounts of *prf1* for mating and further pathogenic development, indicating the presence of additional transcriptional regulators. A potential signaling component for this alternative branch is the novel MAPK Crk1 that can be activated by Fuz7/Ubc5 (Garrido et al., 2004). *crk1*Δ strains are impaired in mating, since they fail to express sufficient amounts of *prf1*. Crk1 mediates *prf1* expression via an unknown transcription factor acting on the upstream activating sequence, a distal promoter element that was previously shown to be involved in carbon source sensing (Hartmann et al., 1999). Thus, transcriptional regulation of *prf1* connects pheromone signaling to nutritional cues in order to fine-tune the complex mating program.

A second way to regulate Prf1 activity is phosphorylation by the catalytic PKA subunit Adr1. As pointed out above, active PKA signaling promotes *mfa1* expression.

Subsequently, it was demonstrated that PREs are necessary and sufficient for cAMP-induced expression, that Adr1 phosphorylates PKA phosphorylation sites in Prf1 in vitro, and that these sites are essential for *a* and *b* gene expression. Thus, active PKA signaling appears to be prerequisite for pheromone-induced expression of mating-type genes (Kaffarnik et al., 2003). This was further supported by the observation that PKA phosphorylation is sufficient to induce a small subset of pheromone-regulated genes including both *b* mating-type genes (Zarnack et al., 2008).

A third way to regulate Prf1 activity is phosphorylation by the pheromone-activated MAPK Kpp2/Ubc3. Initially, it was demonstrated that MAPK phosphorylation sites of Prf1 are functionally important for mating (Müller et al., 1999). Further experiments revealed that the three central sites could be mapped as critical positions that were specifically phosphorylated by Kpp2/Ubc3 in vitro. Intriguingly, MAPK phosphorylation sites in Prf1 are dispensable for *mfa1* expression but essential for expression of *b* mating-type genes, indicating that the MAPK phosphorylation status of Prf1 allows promoter discrimination (Kaffarnik et al., 2003).

Genome-wide expression studies revealed that MAPK phosphorylation of Prf1 is involved in pheromone-regulated expression of 57 genes encoding important regulators such as the b heterodimer and Rbf1. For the majority of genes, pheromone responsiveness was completely lost when MAPK phosphorylation of Prf1 was prevented. However, in some cases pheromone-regulated gene expression was only alleviated. This revealed a novel level of MAPK signaling, suggesting that target genes respond differentially to the MAPK phosphorylation status of Prf1 (Zarnack et al., 2008). A likely explanation would be the presence of one or more coactivator(s) of Prf1, whose interaction might depend on the differential phosphorylation status of Prf1. Thereby, such interaction partners could determine the precise transcriptional outputs.

In summary, the detailed study of the pheromone response in *U. maydis* revealed that mating is orchestrated by a highly sophisticated signal transduction network that consists of at least two branches, the PKA and the MAPK signaling pathway. The complexity of this regulatory system is due to the presence of components that are regulated at various levels or exhibit additional functions at later stages of pathogenic development.

In the future, it will be important to identify missing components of the currently known pathways as well as to uncover additional routes that participate in the network. Moreover, almost one-half of the pheromone-regulated genes encode proteins with unknown function, offering plenty of room for novel discoveries.

THE HOMEODOMAIN TRANSCRIPTION FACTORS

Besides pheromones and cognate receptors, genes encoding the basidiomycete homologues of the a1 and α2 proteins of *S. cerevisiae* are found at the second *MAT* locus, called *MATb* in *U. maydis* (Fig. 5A) (Gillissen et al., 1992) and *MATA* in hymenomycetes (Fig. 5B) (Kües and Casselton, 1992; Stankis et al., 1992). Unlike what is observed in *S. cerevisiae*, the genes are multiallelic in both species and genes for both classes of proteins are found in all versions of the *MAT* locus, because each mate must be able to donate one of each class into a compatible mating. The simplest version of this *MAT* locus is seen in *U. maydis* with just a single pair of divergently transcribed genes. *bE* is the *HD1* gene, and *bW* is the *HD2* gene (Gillissen et al., 1992; Kämper et al., 1995; Kronstad and Leong, 1990). There are a predicted 25 alleles of this gene pair. Each pair

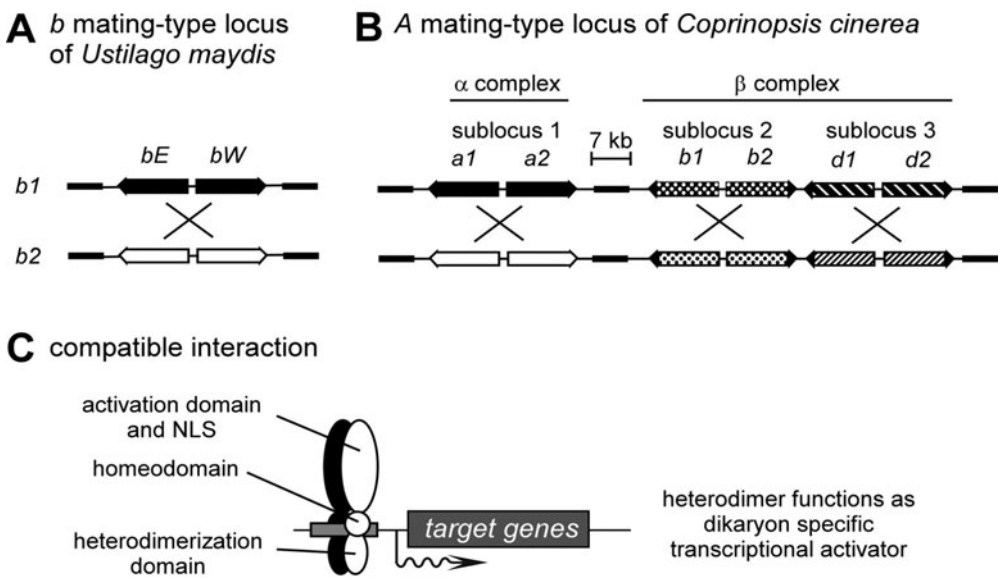


FIGURE 5 Organization of the basidiomycete *MAT* loci encoding homeodomain proteins in *U. maydis* (A) and *C. cinerea* (B). Different fill motifs are used to represent different alleles of genes in both *U. maydis* and *C. cinerea* and paralogous genes in *C. cinerea*. *bE*, *a1*, *b1*, and *d1* are *HD1* genes, and *bW*, *a2*, *b2*, and *d2* are *HD2* genes. Crossed lines indicate compatible gene combinations. (C) Hypothetical heterodimer that results from heterodimerization between compatible HD1 and HD2 proteins.

in a haploid genome is unable to activate development, but different alleles brought together by mating generate two bW/bE heterodimers analogous to the a1/α2 heterodimer of *S. cerevisiae*. The heterodimer is a dikaryon-specific transcription factor that is necessary and sufficient to regulate pathogenic development. Generating pathogenic haploid strains that express an active bW/bE heterodimer revealed that neither cell fusion nor a dikaryotic status is required for the plant-dependent phase of the life cycle (Bölker et al., 1995). The heterodimeric complex binds distinct promoter elements identified in upstream regions of target genes (Brachmann et al., 2001; Romeis et al., 2000). Thus, this transcription factor elicits a defined regulatory cascade including transcriptional activation of secondary transcription factors (Kahmann and Kämper, 2004). Among the targets are important regulators such as the MAPK Kpp6, essential for signaling during plant infection, and Clp1, a protein involved in clamp formation (see below) (Brachmann et al., 2003; Scherer et al., 2006).

Different *bW* and *bE* alleles share DNA sequence similarity in the 3′ regions of the genes, but the promoter region between the genes and the sequences 5′ to those encoding the homeodomains are allele specific, indicating that the 5′ regions determine allelic specificity. The fact that the genes are divergently transcribed means that a nonhomologous DNA region prevents recombination between *bW* and *bE* so that compatible gene combinations are not generated.

The paired *HD1-HD2* genes are also seen in the hymenomycete *MAT*A locus (*a1-a2*, *b1-b2*, and *d1-d2*). In *C. cinerea* the *MAT*A locus, like the *MAT*B locus, has acquired its multiple specificities by gene duplication to give three subloci containing functionally independent (paralogous) genes. In Fig. 5B, the locus illustrated is a hypothetical archetype, in which the compatible gene combinations are shown by crossed lines. None of the actual *MAT*A loci sequenced contain all six genes. The middle pair (*b* gene pair) is always present, but there may be only one or the other of the gene pair at sublocus 1 and sublocus 3 (Pardo et al., 1996). This ensures that at least one active heterodimer can be generated when different alleles of the particular sublocus are brought together in the same cell (Pardo et al., 1996). There are an estimated 4 alleles of sublocus 1, 10 alleles of sublocus 2, and 3 alleles of sublocus 3 (Pardo et al., 1006), sufficient to generate 120 unique combinations, close to the 150 predicted by Day (Day, 1961).

The *MAT*A locus covers approximately 25 kb. As for the *MAT*B locus, integrity of this large complex is maintained by the very dissimilar DNA sequence of different alleles at each sublocus. Significantly, there is a short 7-kb stretch of DNA homology between sublocus 1 and sublocus 2, which would permit the recombination between A genes observed by Day (Day, 1961), hence the designation of Aα and Aβ complexes. In *S. commune*, the Aα and Aβ loci are much further apart (Raper, 1966). The *pab1* and *ade8* genes that flank *MAT*A in *C. cinerea* are found between Aα and Aβ, and the genes at the Aα sublocus are oppositely oriented, suggesting that this arrangement was brought about by an inversion (James et al., 2004). Although the Aα complex has been characterized and corresponds to sublocus 1 of *C. cinerea*, only a single gene of the Aβ complex has been identified, and it is not known how many other A genes exist (Shen et al., 1996). The evolution of multiple alleles went hand in hand with the evolution of a very sensitive dimerization domain in the N-terminal regions of HD1 and HD2 proteins, which permits discrimination between bW and bE proteins encoded by the same locus and by different allelic versions of the locus (Fig. 5C) (Kämper et al., 1995). This dimerization domain is present in the *S. cerevisiae* proteins, but since there are only two proteins, there is no requirement for discrimination (Ho et al., 1994). Studies of *C. cinerea* and *S. commune* confirmed that specificity also lies in the N-terminal domains of the proteins. Swapping the 5′ ends of genes from different subloci or the 5′ ends of allelic genes altered specificity in a predicted way (Kües et al., 1994; Yue et al., 1997). Within the N-terminal domains of the HD1 proteins it is possible to predict two coiled-coil domains, and significantly, these occur at different positions in proteins encoded by different subloci (Banham et al., 1995; Gieser and May, 1994). At the allelic level, substitutions causing charge changes in the corresponding regions of the bE and bW proteins altered dimerization specificity (Kämper et al., 1995).

Heterodimerization of the HD1 and HD2 proteins brings together different functional domains, as illustrated in Fig. 5C. The basidiomycete proteins have long C-terminal domains, unlike their *S. cerevisiae* counterparts, and consistent with the heterodimer being a transcriptional activator, it has been shown that these contain both nuclear targeting signals and potential activation domains (Asante-Owusu et al., 1996; Spit et al., 1998). In *C. cinerea*, both these functional domains are contributed by the HD1 protein and dimerization is essential for nuclear localization. In *S. cerevisiae*, a1/α2 heterodimerization has been shown to play an essential role in DNA-binding specificity (Johnson, 1995), and it seems likely that this also true for the basidiomycete proteins.

HETERODIMER TARGETS AND SEXUAL DEVELOPMENT

So far, we have described the structure of the mating-type loci, the function of the encoded proteins, and the regulatory cascades that are elicited. These regulatory circuits during mating are also connected to various morphological changes in basidiomycetes. Intriguingly, the same mating factors are adapted to the special needs of each fungus during its life cycle.

In higher basidiomycetes, pheromone signaling is not used to coordinate hyphal fusion but is essential to regulate the process of clamp cell formation, as mentioned earlier. In addition, pheromone signaling is essential for nuclear migration, a process that requires the dissolution of the complex dolipore septa that separate the cellular compartments of the monokaryotic mycelium (Giesy and Day, 1965). In *S. commune*, specific glucanases were implicated in septal dissolution (Wessels, 1969), but it is still unknown how pheromone signaling leads to activation of these enzymes. Despite the widespread dissolution of septa, there is no simultaneous exchange of mitochondria and mitochondrial inheritance is unilateral. Casselton and Condit described a mitochondrial mutant (*acu10*) with a defect in oxidative phosphorylation and a distinctive phenotype (Casselton and Condit, 1972). Reciprocal exchange of nuclei on mating the *acu10* mutant to a wild type gave rise to two morphologically discrete dikaryons. Subsequent work established that mixed mitochondrial populations exist at the site of hyphal fusion, and this can lead to recombination of mitochondrial genome markers (Baptista-Ferreira et al., 1983), but there is rapid segregation of different mitochondrial types.

The hymenomycete dikaryon can exist for an indefinite period but gives rise to highly differentiated fruiting bodies in response to the right environmental cues. The mushroom fruiting body is complex, and there are several stages in its development (Moore, 1996). Localized branching gives rise to hyphal knots that then differentiate cap and stipe tissues. Light is required for karyogamy, meiosis, and basidiospore formation. Light is essential also for maturation of the fruiting body, which involves expansion of the cap and elongation of the stipe. The fruiting body is short-lived, and the cap rapidly autolyzes, releasing the black basidiospores. In the absence of light, so-called "dark stipes" are produced, which are composed of basal tissue with rudimentary stipes and caps at the apex (Buller, 1931). The complete developmental sequence has been reviewed in detail by Kües (Kües, 2000).

Isolating mutants defective in morphogenesis has been simplified by the use of special strains with self-compatible mutations in both sets of *MAT* genes. Early mutation studies of the *MAT* genes of *C. cinerea* and *S. commune* focused on the large numbers of alleles and the possibility of generating new alleles by mutation. Mutations in both *A* and *B* genes proved to be extremely rare and required strong selection techniques (Day, 1963; Haylock et al., 1980; Raper, 1966). These were not mutations to new alleles; all were dominant and resulted in self-compatibility and constitutive expression of the morphogenetic pathway regulated by the mutant gene (i.e., clamp cells or nuclear migration). Mutations in *MATA* proved to be the result of in-frame fusions between *HD1* and *HD2* genes to give chimeric genes encoding a minimal heterodimer with all the functional domains required to activate development (Asante-Owusu et al., 1996; Kües and Casselton, 1992; Pardo et al., 1996). Mutations in *B* genes result either in mutant pheromones that can activate self receptors (Fowler et al., 2001), in receptor genes that generate constitutively active receptors (Olesnicky et al., 1999), or in receptors that are activated by self pheromones (Olesnicky et al., 2000). The combination of an *A* and *B* mutation in the same genome gives haploid strains that mimic in nearly all respects a true dikaryon. *AmutBmut* strains produce uninucleate oidia that can be used for mutagenesis, and since the nuclei in each cell of the resulting dikaryon are identical, it is possible to identify recessive mutations affecting what would normally be a developmental phase with two genetically different nuclei. These *AmutBmut* strains are fertile and can been used to select for mutations affecting all stages of sexual development, from dikaryon formation to meiosis. The mutants most commonly used for these studies were isolated by Swamy and colleagues (see also chapter 7 for mutants with defects in meiosis and sporulation) (Swamy et al., 1984).

Interesting mutants affecting dikaryon formation are *hmg1*, *clp1*, and *pcc1*. *hmg1* encodes a transcription factor with an HMG domain similar to Prf1 of *U. maydis*. Hmg1 is necessary for the nuclear migration that initiates dikaryon formation in *C. cinerea*, and mutants can donate nuclei only to a wild-type partner, making dikaryotization unilateral. *hmg1* mutants can, however, form normal dikaryons with fused clamp connections. As described earlier, pheromone signaling in hymenomycetes is implicated in both nuclear migration and clamp cell fusion, and it seems likely that different transcription factors are targeted to bring about these different outputs. It is probable that Hmg1 is essential for nuclear migration and that a homologue of Prf1, which can be found in the genome sequence, is essential for clamp cell fusion (C. Aime, M. J. Milner, and L. A. Casselton, unpublished data.). A putative target of the A protein heterodimer, *clp1*, was identified using the *AmutBmut* background to detect mutants unable to form clamp connections. The Clp1 protein is essential for activating the clamp cell pathway and is normally induced only following a compatible *A* gene (heterodimer) interaction. However, if *clp1* is expressed from a constitutive promoter, the dependence on heterodimer function is bypassed (Inada et al., 2001). An orthologue of this gene was subsequently isolated from *U. maydis* and, as described below, was actually shown to be a direct target of the bW/bE heterodimer. Notably, both the *C. cinerea* and the *U. maydis* genes have a promoter motif that resembles that of the a1/α2 heterodimer of *S. cerevisiae* (Scherer et al., 2006). Pcc1 is another member of the HMG domain family of transcription factors (Murata et al., 1998). The *pcc1* mutation permits fruiting without mating, and hyphae have unfused clamp cells. Significantly, *ppc1* mutants are unable to fuse their clamp cells or permit nuclear migration, suggesting that Pcc1 is required for pheromone signaling and in some way inhibits the clamp cell pathway. The functions of the *A* and *B* *MAT* genes may well be antagonistic. The heterodimer is essential for tip cell growth and nuclear division, whereas the pheromone signal causes temporary cell cycle arrest.

As easy as it has proved to isolate mutants affected in dikaryon or fruiting-body formation (Kües, 2000), understanding how gene functions fit into the complete developmental sequence requires a much broader look at gene expression. With the complete genome sequence available for *C. cinerea*, microarray analyses of defined stages in development and the effects of specific defects promise to provide relevant clues.

Although the same mating-type components exist in *U. maydis*, their function in morphogenesis is different from those in higher basidiomycetes. In *U. maydis*, the first morphological transition is the switch from sporidial growth to a filamentously growing conjugation tubes. Upon initial stimulation, pheromone perception as well as secretion is polarized at the hyphal tips (Fuchs et al., 2005). Growing hyphae use the spatial cue of the pheromone gradient for directed growth towards the mating partner. This is crucial for fusion at the hyphal tips, as was very elegantly demonstrated by Snetselaar and colleagues (Snetselaar et al., 1996).

The second morphological transition occurs after cell fusion, when the gene products of the *b* mating-type locus trigger the formation of filamentously growing dikaryotic hyphae. These infectious hyphae grow with a defined axis of polarity: they expand at the apical tip and insert retraction septa at the distal pole. The latter results in the formation of regularly spaced empty sections at the rear end of the hyphae (Steinberg et al., 1998). Tip expansion is mediated by the Spitzenkörper, a vesicle supply center that is supported with growth supplies by active transport along the actin and microtubule cytoskeleton for short-distance and long-distance transport, respectively (Steinberg, 2007). Recently, it has been suggested that besides endosomes, mRNAs are important molecular cargos that might support local translation at the hyphal tip (Zarnack and Feldbrügge, 2007). The notion is based on the observation that the RNA-binding protein Rrm4 is involved in determining the axis of polarity in b-dependent hyphae (Becht et al., 2005, 2006). This posttranscriptional regulator shuttles bidirectionally along microtubules and recognizes distinct mRNAs in vivo, suggesting that long-distance transport of mRNAs is crucial for hyphal growth (Feldbrügge et al., 2008; Zarnack and Feldbrügge, 2007).

A third morphological transition is the formation of specialized infection structures on the plant surface. Formation of these so-called appressoria is regulated by components of the pheromone-regulated MAPK module (Brachmann et al., 2003; Müller et al., 2003b). After penetration of the epidermis, the cell cycle arrest is released. Inside the plant, the fungus proliferates and forms a multicellular mycelium. Essential for cell division of the dikaryon in planta is Clp1, a factor involved in clamp formation that antagonizes the function of the b heterodimer. Intriguingly, *clp1* is a direct target of the b heterodimer, although Clp1 expression is confined to later, plant-associated stages by posttranscriptional mechanisms (Scherer et al., 2006). Thus, regulatory proteins encoded at the *b* mating-type locus are also essential to prepare later steps of pathogenic development (see chapter 39).

EFFICIENCY OF MATING SYSTEMS

Most evolutionary biologists would agree that sexual reproduction is advantageous because it is crucial to maintain genetic diversity, enhance adaptive evolution, and eliminate deleterious mutations. The mating systems evolved by basidiomycetes are the most efficient in the fungal kingdom. Mating systems act primarily to promote outbreeding, but at the same time they also restrict sib-compatibility (mating between cells derived from the same fruiting body). With just two mating types in ascomycete fungi, sib-compatibility is 50%, but with only two alleles in the population outbreeding potential is also 50%. In tetrapolar mating systems, with four mating types segregating, there is greater restriction on sib-compatibility (25%), but with just two allelic versions of both loci, outbreeding potential would be similarly restricted. By evolving multiallelic *MAT* genes, sib-compatibility remained the same, but outbreeding potential increased rapidly with increasing numbers of alleles, a strong driving force that has led to remarkable numbers of mating types in some species. In *C. cinerea*, it was estimated from population studies by Day (see Raper, 1966) that there are some 150 allelic versions of *MATA* and 79 of *MATB*, giving more than 12,000 mating types. Collections worldwide of *S. commune* suggested as many as 288 *MATA* alleles and 81 *MATB* alleles (giving more than 20,000 mating types [Raper, 1966]). Outbreeding potential can be calculated from the formula $(n_a n_b - n_a - n_b + 1)/n_a n_b$ (Koltin et al., 1972). In *U. maydis*, outbreeding potential is low (48%). With three alleles of *MATa*, the outbreeding potential of *S. reilianum* is already greater than that of *U. maydis* even with just the five known alleles of *MATb*. In *C. cinerea* and *S. commune* outbreeding potential is more than 90%.

BIPOLAR SPECIES AND SEX CHROMOSOME EVOLUTION

Molecular analysis of bipolar basidiomycete species suggests that these have all arisen from tetrapolar ancestors. In heterobasidiomycetes, such as smuts and *C. neoformans*, the *MAT* loci are very large and contain genes encoding both the pheromones and their receptors and the homeodomain proteins (Bakkeren and Kronstad, 1994). In homobasidiomycetes, the single *MAT* locus encodes only the homeodomain proteins and while the pheromone and receptor genes are present in the genome, they are physically unlinked to *MAT*, as they are in tetrapolar species, and have ceased to be mating-type determinants (James et al., 2006). Figure 6 illustrates the organization of *MAT* loci in three bipolar species, *Ustilago hordei*, *C. neoformans*, and *Coprinellus disseminatus*.

U. hordei, the barley smut, is a close relative of *U. maydis*, and its two alleles of the *MAT* locus are designated *MAT-1* and *MAT-2*. The *MAT* locus was first identified using genes from *U. maydis* as hybridization probes (Bakkeren et al., 1992), and further analyses then established that the *a* and *b* mating-type genes were physically linked (Bakkeren and Kronstad, 1994). Each version of *MAT* contains a divergently transcribed *bE* and *bW* gene pair at one end of the locus as well as the orthologues of *pra* and *mfa*, the receptor and pheromone genes, at the other end. Surprisingly, between these two sets of genes is some 450 to 500 kb of DNA sequence within which there is complete crossover suppression (Fig. 6A). Sequencing of the 500-kb region between *bE1* and *bW1* as well as *pra1* and *mfa1* at the *MAT-1* locus identified some 47 protein-coding genes, none of which was associated with pathogenicity or mating (Bakkeren et al., 2006). The analysis revealed that about 50% of the sequence was composed of repetitive elements containing islands of the protein-encoding genes. By comparing the sequences of *MAT-1* and the regions around the *a* and *b* genes with corresponding regions of the *U. maydis* genome, it was apparent that there was retained synteny around the *MAT* genes themselves. However, the large accumulation of repetitive elements in *MAT1* had led to inversions and transpositions of other genes. Such rearrangements together with sequence diversification would lead to the lack of sequence homology that would enable homologous recombination.

In *C. neoformans*, the two alleles of *MAT*, known as *MATa* and *MATα*, cover ca. 100 kb and contain not only the pheromone and receptor genes and the homeobox genes, but also at least 20 genes encoding proteins of the pheromone signaling cascade such as *STE11*, *STE20*, and *STE12*, as well as genes of unrelated function (Hull et al., 2005; Hull and Heitman, 2002). *C. neoformans* is similar to *S. cerevisiae* in having just a single allele of the *HD1* and *HD2* genes. The *HD1* gene (*SXI1α*) in the *MATα* locus and the *HD2* gene (*SXI2a*) in the *MATa* locus are sufficient to generate a single heterodimer following cell fusion (Fig. 6B). There is no sexual advantage in retaining both members of the gene pair in each locus as occurs in *U. hordei*. Similar to *U. hordei*, however, is the evidence of inversions, translocations, sequence diversification, and repetitive elements contributing to the overall crossover suppression in this sex-determining region of the chromosome. Interestingly, there are two hot spots for recombination flanking *MATa* and *MATα*, enabling the entire gene complex to be recombined into different genetic backgrounds during sexual reproduction (Hsueh et al., 2006).

There are obvious parallels between the evolution of highly polymorphic sex-determining regions of basidiomycete fungi and the sex chromosomes of plants and animals (Frazer and Heitman, 2005). In *M. violaceum*, another bipolar smut, the chromosomes carrying the *MAT* loci are distinguishable by an obvious length polymorphism, again, a common feature of sex chromosomes in other organisms (Giraud et al., 2008).

In heterobasidiomycetes, pheromone secretion is essential for mate attraction and cell fusion, but in hymenomycetes, as seen in *C. cinerea* (Fig. 1A), fertilization involves random hyphal fusion, which is mating-type independent. All the available evidence indicates that pheromone signaling is activated only after cell fusion. In bipolar *C. disseminatus*, the single *MATA* locus contains

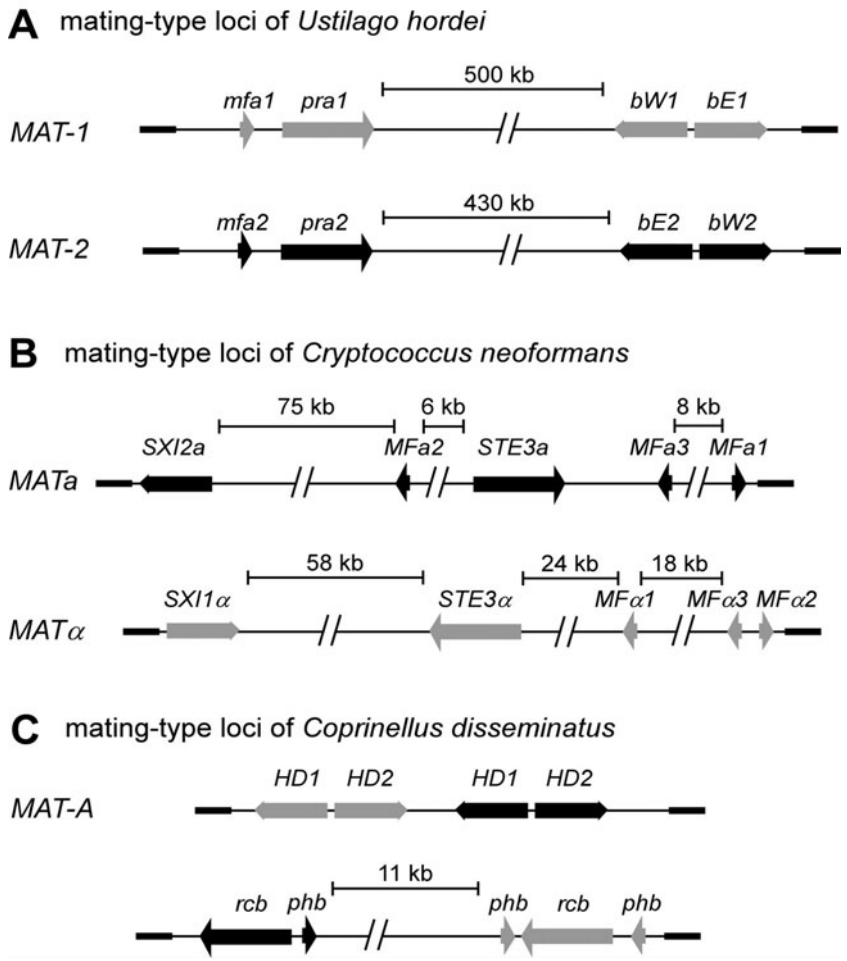


FIGURE 6 Organization of mating-type loci of bipolar species *U. hordei* (A), *C. neoformans* (B), and *C. disseminatus* (C). Pheromones, receptors, and homeodomain proteins are indicated with the same symbols as in Fig. 3 and 5. Large gaps between mating-type loci are indicated with the distance in kilobases. Pheromone and receptor genes shown for *C. disseminatus* are unlinked to *MATA*.

two pairs of genes encoding the dissimilar homeodomain proteins but no pheromone or receptor genes. Dimerization between HD1 and HD2 proteins following cell fusion is apparently sufficient for the recognition of a compatible mating. However, the dikaryon that develops has binucleate cells and fused clamp connections, indicating that pheromone signaling still plays an essential role in mating (James et al., 2006). The pheromone and receptor genes reside on an unlinked chromosome, just as they do in tetrapolar species, but it is likely that they are activated once heterodimer function has established that a compatible cell fusion occurred (Fig. 6C). James et al. (2006) suggest that pheromone signaling might be constitutive in bipolar species as a result of having compatible combinations of pheromones and receptors. The genes must be self-compatible, but it is unlikely that they are constitutively active, since this causes a very debilitating phenotype when induced experimentally in *S. commune* (Raper, 1966). As with smuts, it is likely that bipolar hymenomycetes evolved from tetrapolar forms, but this has not been achieved by locus fusion. Having dispensed with the need for pheromone signaling in mate attraction, the activity of the pheromone genes is required only after cell fusion (fertilization), and it seems likely that the way in which they are regulated was also modified.

Of the self-compatible hymenomycetes, some, like *Agaricus bisporus*, are secondarily homothallic and retain the need for different mating types. *A. bisporus* is a bipolar species, and as the name implies, it produces just two basidiospores on each basidium. Two of the meiotically produced nuclei enter each spore, and these are generally compatible so that spore germination gives rise directly to a fertile mycelium. Rare three- and four-spored basidia arise with nuclei in which the mating-type genes have segregated and compatible cell fusion becomes essential to establish fertility (Elliott, 1978). The *A. bisporus MATA* locus has been partially characterized and contains at least a single pair of *HD1-HD2* genes (Y. Li, L. A. Casselton, and M. Challen, unpublished data). Other species are truly self-compatible, like *Agaricus subfloccosus*. Preliminary analysis of this species led to amplification of homeobox genes analogous to those in *A. bisporus* (S. Burrow, L. A. Casselton, and M. P. Challen, unpublished data), indicating that true homothallism, as in ascomycete species such as *Sordaria*

macrospora (Pöggeler and Kück, 2000) and *Aspergillus nidulans* (Galagan et al., 2005), results from sequestering compatible sets of mating-type genes within the same genome.

MAT EVOLUTION IN THE DIKARYA

The evolution of heterothallism required the sexual cycle to be interrupted close to its initiation and key genes to be localized to a special sex locus (now termed *MAT*) in such a way that fertilization was essential to bring the genes back together. The key genes that initiate sexual development in most fungal species are those required for pheromone signaling, and it is the activities of these genes that we see regulated at *MAT*. The two ways in which *MAT* genes may effect this regulation is illustrated in Fig. 7.

In all ascomycetes, as described earlier for *S. cerevisiae*, the two types of haploid cells synthesize different pheromones and receptors, requiring the coordinated regulation of a subset of genes in a *MAT*-dependent fashion (Herskowitz, 1988). The functional significance of having these two classes of pheromones is not known. In *S. cerevisiae*, the α-domain protein activates expression of the *MATα* cell-specific genes whereas the *MATa* cell-specific genes are expressed constitutively. In other yeast species, however, such as *C. albicans* and *Yarrowia lipolytica*, which diverged from the *S. cerevisiae* lineage many millions of years ago, *MATa* cell-specific gene expression is not constitutive but is dependent on activation by another transcription factor encoded at *MATa*, an HMG domain protein (Butler, 2007) (Fig. 7 and 8). *S. cerevisiae* and close relatives have lost this gene and had to evolve the alternative way of regulating the genes shown in Fig. 2. The orthologues of the α-domain and HMG-domain genes are found in the *MAT* loci of all filamentous ascomycete fungi (Fig. 8) (Coppin et al., 1997; Debuchy and Coppin, 1992) and are the only genes found at the *MAT* loci of dothideomycete fungi such as *Cochliobolus heterostrophus* (Turgeon et al., 1993). It is likely that their functions are the same as those of the yeast genes as indicated in Fig. 7.

In basidiomycetes, the development of a tetrapolar mating system demanded multiple alleles of *MAT* genes in order to make outbreeding efficient, and this would have been impossible if *MAT* genes still regulated the many genes necessary for differential pheromone and receptor gene expression. The pheromone and receptor genes became dedicated *MAT* determinants, and as a consequence only one class of pheromone and receptor could be encoded (Fig. 7). As described earlier, these are members of the lipopeptide **a**-pheromone and Ste3p receptor families (Kurjan, 1993).

Figure 8 summarizes likely steps in the evolution of different forms of the *MAT* loci of the Dikarya and is an extension of ideas put forward by Casselton (Casselton, 2008). The *MAT* locus of the ancestral Dikarya was likely to be similar to that of the present-day Dothideomycetes, an idiomorphic *MAT* locus encoding either an α-domain transcription factor or an HMG-domain transcription factor (Fig. 8, steps 1 and 2). In Sordariomycetes, as described above, two other genes were acquired by one idiomorph but not for pheromone gene regulation (Fig. 8, step 3). Yeasts acquired genes encoding the HD1 and HD2 homeodomain proteins as seen in *C. albicans*, where the function of these proteins is only required after mating cell fusion (Fig. 8, step 4). Some species lost the HMG-domain gene, as seen in *S. cerevisiae* and close relatives, with additional functions assigned to the α2 protein (Fig. 8, step 5). In basidiomycetes the α-domain and HMG-domain genes became obsolete, and pheromone and receptor genes elsewhere in the genome were recruited to create a second unlinked *MAT* locus. Amplification and diversification of *HD1* and *HD2*

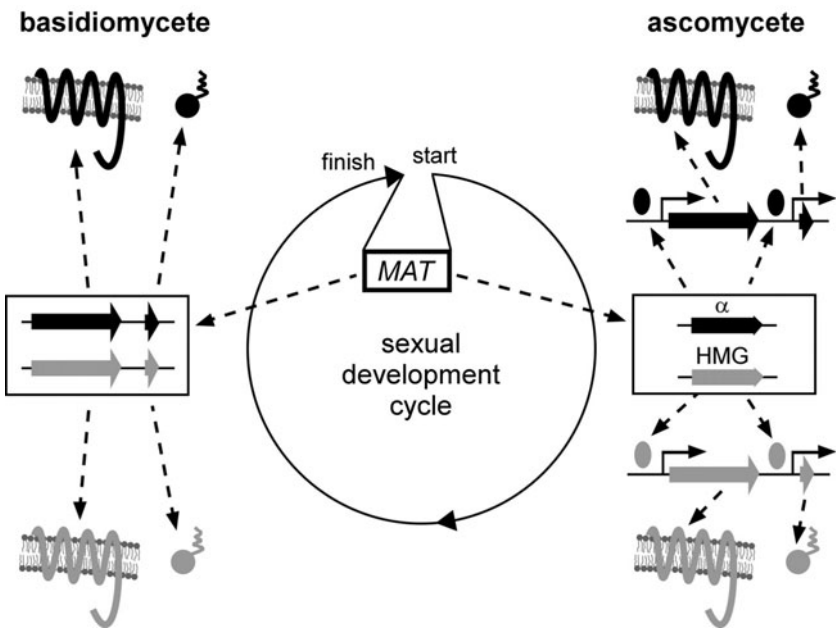


FIGURE 7 Comparative regulation of the sexual cycle in heterothallic basidiomycetes and ascomycetes. Evolution of heterothallism resulted in critical genes required to initiate the sexual cycle being localized to a *MAT* locus. In ascomycetes, the genes at *MAT* are translational activators that regulate differential expression of pheromone precursor and pheromone receptor genes and the many other genes required for processing the pheromones to active species. In basidiomycetes, the pheromone precursor and pheromone receptor genes are localized to the *MAT* locus.

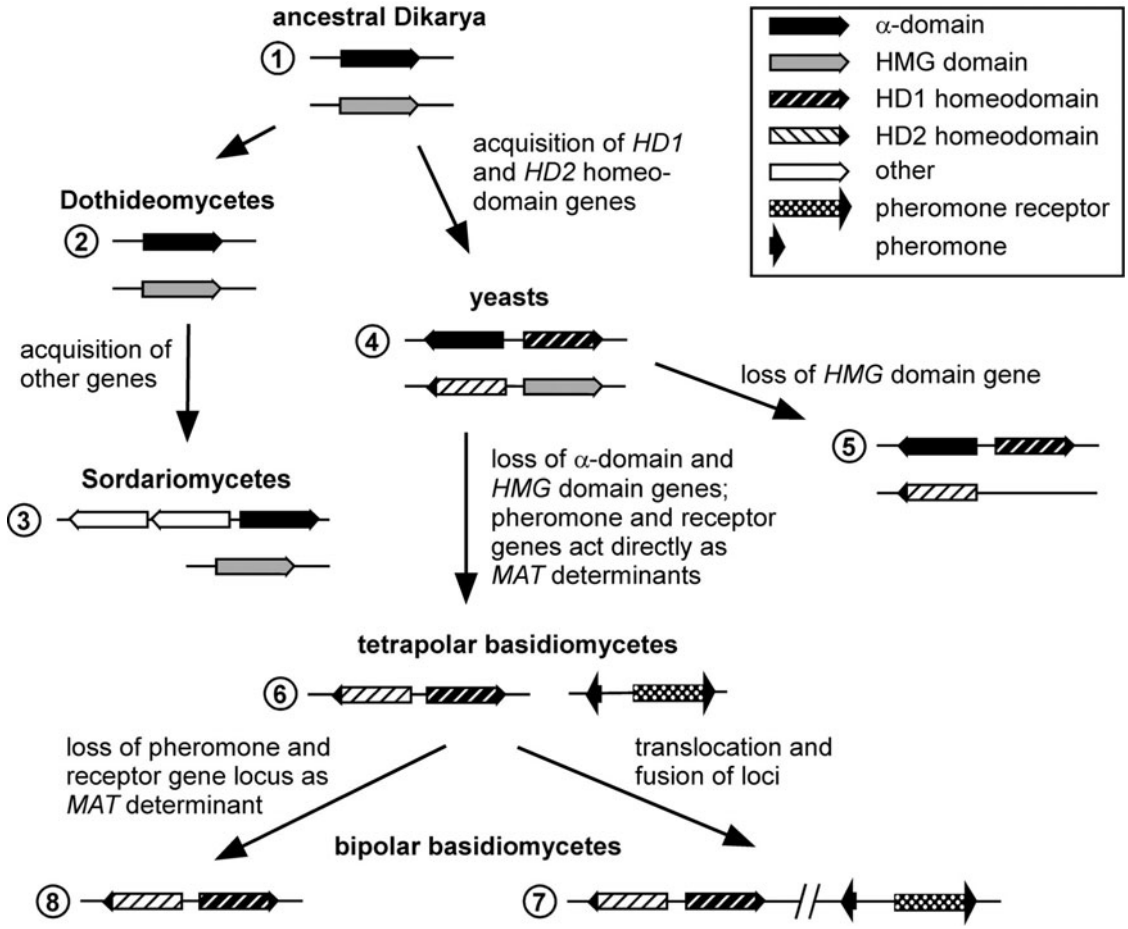


FIGURE 8 Suggested steps in the evolution of *MAT* loci in ascomycetes and basidiomycetes. Refer to the text for details.

genes were needed to create multiallelic genes as found in *U. maydis* (Fig. 8, step 6). Further amplification of genes at both *MAT* loci gave rise to the paralogous gene complexes found in hymenomycetes. Conversion of a tetrapolar mating system to a bipolar one was either by translocation and fusion of the *MAT* loci to generate the large sex loci seen in *C. neoformans* and *U. hordei* (Fig. 8, step 7) or by losing the receptor and pheromone locus as a mating-type determinant as occurred in bipolar hymenomycetes (Fig. 8, step 8).

SUMMARY

The model basidiomycete species that we have chosen to describe illustrate just how conserved the components of the mating pathway are, despite the very different lifestyles these fungi have adopted. Peptide pheromones and their cognate receptors are necessary for cell-cell communication, whether it be to signal over a distance on a plant leaf to attract a compatible mating partner as occurs in *U. maydis* or for communication between adjacent cells to promote nuclear migration and clamp cell fusion in the dikaryon, as seen so clearly in *C. cinerea* and other hymenomycetes. In addition, successful mating requires two distinct classes of homeodomain proteins to generate a dikaryon-specific transcription factor. Elegantly, the activity of this transcriptional regulator is determined at the level of heterodimerization. Thus, an active heterodimer is formed only after fertilization, because it depends on mating partners bringing together compatible versions of the two proteins.

As we have described, studies of bipolar heterobasidiomycete species in particular, such as *U. hordei* and *C. neoformans*, have sparked an interest in how sex chromosomes evolve. Obvious parallels can be found in the way in which fungi, plants, and animals have evolved unique sex-determining regions, in particular the common finding of remnants of transposable elements and the rearrangement and sequence diversification of genes.

The dikaryons of our model species, though dependent on the same signaling processes and similar transcription factors, are seen to differentiate very different structures. The pathogenic *U. maydis* dikaryon engages with its host plant and develops specialized structures for host invasion and proliferation within the plant before inducing tumors full of sexual spores. In contrast, the hymenomycete dikaryon is a long-lived mycelium that can differentiate complex fruiting bodies while continuing to live vegetatively. Depending on the species, it can colonize many different substrates and even form a nonpathogenic association with tree roots, as occurs with ectomycorrhyzal species. Understanding the latter interaction is the goal of recent genome research involving *Laccaria laccata* (Martin et al., 2008), and it will be particularly interesting to see

what parallels are found in the pathogenic and symbiotic relationship between host and fungus and how signaling pathways have been adapted for this purpose. Finally, we may note that the dikaryophase of different species may also be multinucleate or clampless or even convert to diploidy (Anderson and Kohn, 2007); clearly *MAT* gene function has accommodated to wide morphological variation.

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ASEXUAL SPORULATION

IX

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Cellular and Molecular Biology of Filamentous Fungi
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Regulation of *Aspergillus* Conidiation

MIN NI, NA GAO, NAK-JUNG KWON, KWANG-SOO SHIN, AND JAE-HYUK YU

OVERVIEW OF CONIDIATION

The genus *Aspergillus* represents the most common fungi found in the environment. Many *Aspergillus* species are beneficial to humans, but they also include serious animal and plant pathogens. Moreover, almost all *Aspergillus* species can produce one or more toxic secondary metabolites (see Yu and Keller, 2005). All aspergilli produce asexual spores (conidia) as the main means of dispersion, and asexual sporulation (conidiation) and the production of certain mycotoxins are closely correlated (reviewed by Calvo et al., 2002, and Yu and Keller, 2005). Of all *Aspergillus* species, *Aspergillus nidulans* is by far the most thoroughly studied (Martinelli, 1994). The ease of genetic analyses and the availability of numerous experimental tools and genome sequences make *A. nidulans* an excellent model system for various biological questions.

Conidiation of *Aspergillus* provides a well-established model system for studying spatial and temporal regulation of gene expression, specialized cellular differentiation, intercellular communications, and interspecies gene conservations. The asexual reproductive cycle can be divided into two main phases: vegetative growth and development. The growth phase involves germination of a conidium and formation of an undifferentiated network of interconnected hyphal cells that form the mycelium. After a certain period of vegetative growth, under appropriate conditions, some of the hyphal cells stop normal growth and begin asexual development, which includes conidiophore formation and conidial maturation (reviewed by Adams et al., 1998). The life cycle of a fungal spore itself can be divided into a series of stages: formation, maturation, dormancy, and germination (Griffin, 1994). In detail, conidiophore formation starts from thick-walled hyphal cells (foot cells), which extend into the air to produce stalks, followed by the formation of a multinucleate vesicle. In a subsequent budding-like process, two layers of uninucleate reproductive cells, the metulae and phialides, are formed on top of the vesicle. Chains of conidia arise from repeated asymmetric mitotic division of phialides. The final multicellular conidium-bearing structures are called conidiophores (Fig. 1A and B) (Mims et al., 1988; Yu et al., 2006; Ni and Yu, 2007).

After the spore is initially formed, it must undergo the maturation process. The new discovery of a novel master regulator of spore maturation that represses development (discussed below) suggests that conidium maturation is a separate phase distinct from initiation of development. In the absence of nutrients, mature spores enter an extended quiescence, i.e., dormancy, until the environmental conditions are appropriate for germination. Conidium maturation is a defined differentiation process occurring after the formation of the septum between the phialide and the newly formed conidium, resulting in the delimitation of a conidium from the phialide. It is not a well-understood process and involves changes in cell wall structure and chemical composition. Cell wall modification associated with the maturation of conidia occurs in three stages as shown in Fig. 1C (Sewall et al., 1990a). At stage I, conidia are completely separated from the phialide and they contain two layers of cell wall (outer C1 and inner C2 layers), similar to the hyphal cell wall (two layers of P1 and P2). During stage II, the C2 layer condenses and C1 becomes the crenulated rodlet layer. Furthermore, the C1 layer is mostly separated from C2, contacting only C2 projections. In the following stage, stage III, a third wall layer, C3, forms between C1 and C2. The innermost wall, C4, which forms last, is thought to be produced by the deposition of wall material from plasma membrane-associated vesicles in the spore itself. Transmission electron microscopic analysis has shown that outer layer C1 is electron dense, while the inner three layers, C2, C3, and C4, are electron light (Weisberg and Turian, 1971). The four-layer cell wall of mature conidia renders them impermeable, which is required for spore protection and dormancy under harsh conditions.

The composition of chemical components in conidia also changes during conidium maturation. In particular,

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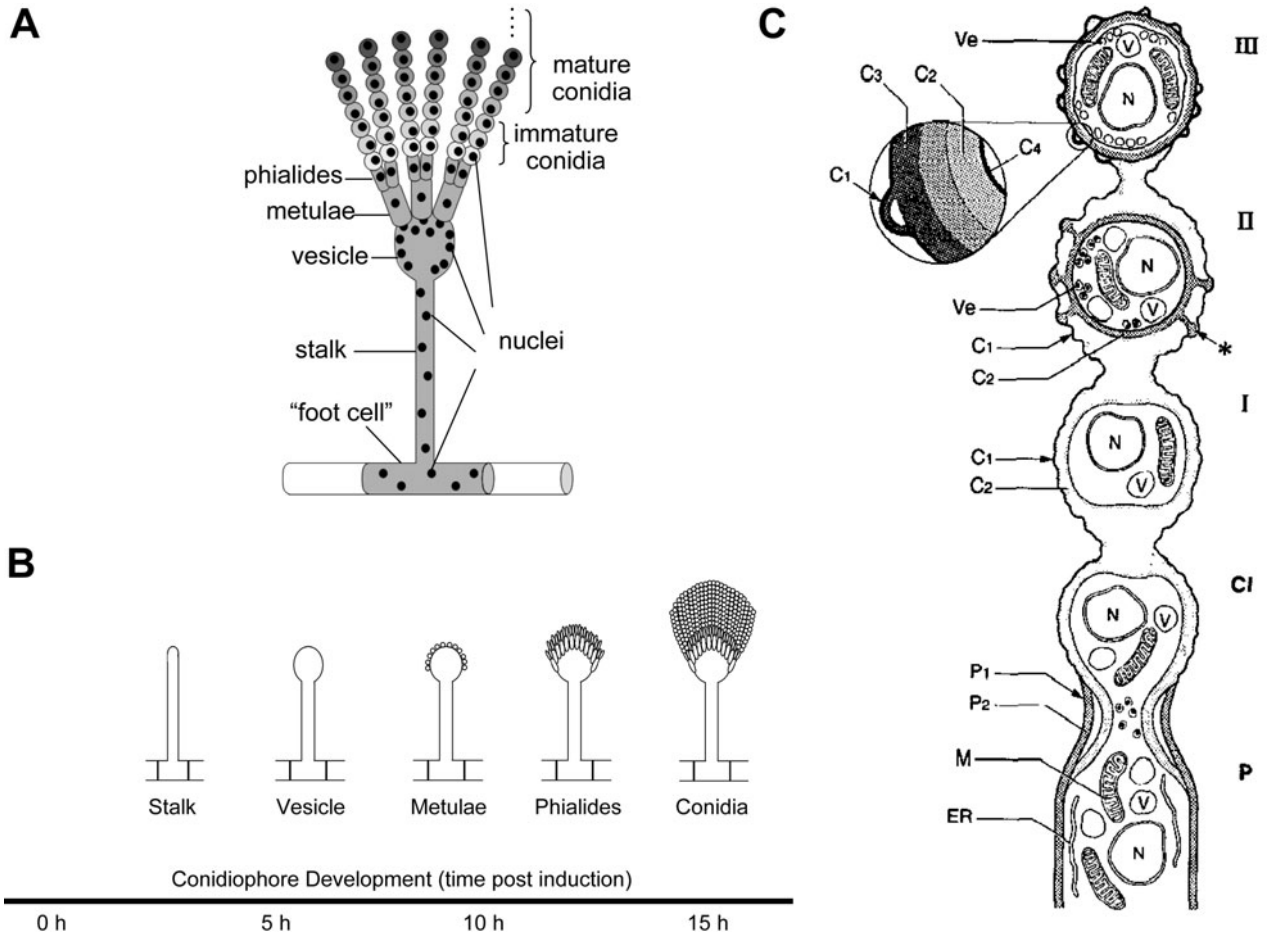


FIGURE 1 Conidiophore formation and spore maturation. (A) A simplified diagram of a conidiophore (reproduced from Ni and Yu, 2007, with permission). (B) The stages of conidiophore development in *A. nidulans* (adapted and modified from Mah and Yu, 2006). (C) The stages of conidium maturation (reproduced from Sewall et al., 1990a, with permission of the publisher). Each conidium and the phialide (P) contains a nucleus (N), mitochondria (M), endoplasmic reticulum (ER), vacuoles (V), and vesicles (Ve). Three stages (I, II, and III) of conidium maturation are shown after a conidium initial (CI) is delimited from the phialide. The phialide contains two cell wall layers: P1 and P2. The CI and conidium at stage I also contain two cell wall layers: C1 and C2. At stage II, the C2 layer condenses to form projections (*) in contact with C1. During stage III, the conidium becomes mature, containing four cell layers: C1 through C4.

accumulation of a large amount of trehalose is the best-characterized process during this phase. Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside, a nonreducing disaccharide) is produced by a wide variety of organisms including bacteria, fungi, protozoa, plants, and invertebrates. It has been shown to serve as an energy source and a protectant against various environmental conditions including desiccation, dehydration, heat, cold, and oxidation (Elbein et al., 2003). The important aspects of dormancy and germination of conidia are related to the nature of the restraint on trehalose catabolism. In *A. nidulans*, conidia contain high levels (up to 15% of the dry mass) of trehalose, and trehalose is degraded very quickly during spore germination (Lingappa and Sussman, 1959; Fillinger et al., 2001). Spores also require large amounts of trehalose for long-term survival. Deletion of the *tpsA* gene (encoding trehalose-6-phosphate, which catalyzes the first step of trehalose biosynthesis) leads to failure in accumulating trehalose and a rapid loss of viability of conidia upon storage (Fillinger et al., 2001).

The entire process of vegetative growth, conidiophore formation, and spore maturation is genetically programmed. Timberlake (1980) carried out RNA-DNA hybridization experiments to estimate the number of diverse transcripts present during different life phases and found that (i) ~6,000 diverse sequences are present in hyphae; (ii) ~1,000 diverse sequences are present in conidiating cultures (containing hyphae, conidiophores, and spores) but not in hyphae and mature conidia; and (iii) ~200 diverse sequences are present in mature conidia only. These data indicate that groups of distinct genes are responsible for each life phase and further imply that conidium maturation is a distinct phase that follows spore formation. The primary aim of this chapter is to discuss our current understanding of how conidiation in *Aspergillus* is regulated, with emphasis on the model fungus *A. nidulans*.

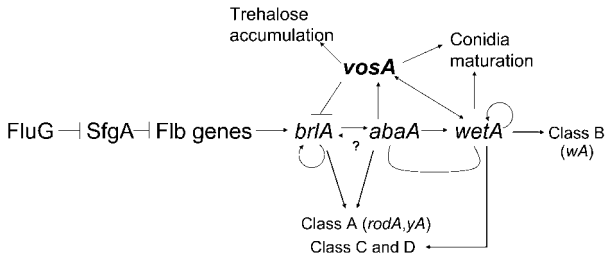


FIGURE 2 Regulatory pathways of conidiation in *Aspergillus nidulans*. *brlA*→*abaA*→*wetA* constitutes the central regulatory pathway controlling conidiation. These genes cooperatively activate other genes (class A through D) responsible for the morphogenesis of conidiophores. FluG and Flb genes are upstream genes required for activation of conidiation (reviewed by Adams et al., 1998). The *fluG* repressor SfgA acts as a negative regulator of conidiation (Seo et al., 2006). VosA functions as a master regulator of spore maturation, which couples sporogenesis and trehalose biogenesis to complete spore maturation and confers negative-feedback regulation of developmental specific genes by repressing the expression of *brlA* (Ni and Yu, 2007).

CENTRAL REGULATORY PATHWAY OF ASEXUAL DEVELOPMENT IN *ASPERGILLUS*

As mentioned above, conidiation in *A. nidulans* is a precisely timed and genetically programmed event. A key and essential step for conidiophore development is activation of the *brlA* gene (Fig. 2) (Clutterbuck, 1969; Adams et al., 1988). Further genetic and biochemical studies identified *abaA* and *wetA*. The *abaA* gene encodes a developmental regulator that is activated by *brlA* during the middle stages of conidiophore development after differentiation of metulae (Andrianopoulos and Timberlake, 1991, 1994). The *wetA* gene functions in the late phase of conidiation for the synthesis of crucial cell wall components (Sewall et al., 1990a; Marshall and Timberlake, 1991). These three genes (*brlA*→*abaA*→*wetA*) have been proposed to define a central regulatory pathway that acts in concert with other genes to control conidiation-specific gene expression and determine the order of gene activation during conidiophore development and spore maturation (Mirabito et al., 1989; reviewed by Adams et al., 1998).

BrlA Is an Essential Activator of *Aspergillus* Conidiation

The *brlA* null mutants, including *brlA1* (Fig. 3A) (Clutterbuck, 1969; Boylan et al., 1987), show bristle-like structures on the surface of colonies. They produce the elongated stalk (about 20 to 30 times the normal length) and fail to develop vesicles or any other subsequent structures.

The *brlA* gene was cloned by complementation of a recessive mutant (Johnstone et al., 1985; Boylan et al., 1987). The *brlA* gene has a complex structure and encodes two overlapping transcription units, *brlA*α (2.1 kb) and *brlA*β (2.5 kb) (Prade and Timberlake, 1993). Both are expressed as early as 10 h after developmental induction, when vesicles start to form (Fig. 3B and C). Mutations that block the expression of either transcript alone cause abnormal development (Prade and Timberlake, 1993). The *brlA*α^+;*brlA*β^- mutant produces abnormal sterigmata without conidial chains, and secondary stalks and vesicles can be generated from a fraction of the sterigmata in the primary conidiophores. The *brlA*α^-;*brlA*β^+ mutant produces nearly normal stalks and vesicles but abnormal sterigmata without conidia chains. Multiple copies of either *brlA*α or *brlA*β can compensate for loss of the other gene, indicating that the *brlA*α and *brlA*β transcription units are individually required for normal development but the products of each gene have redundant functions.

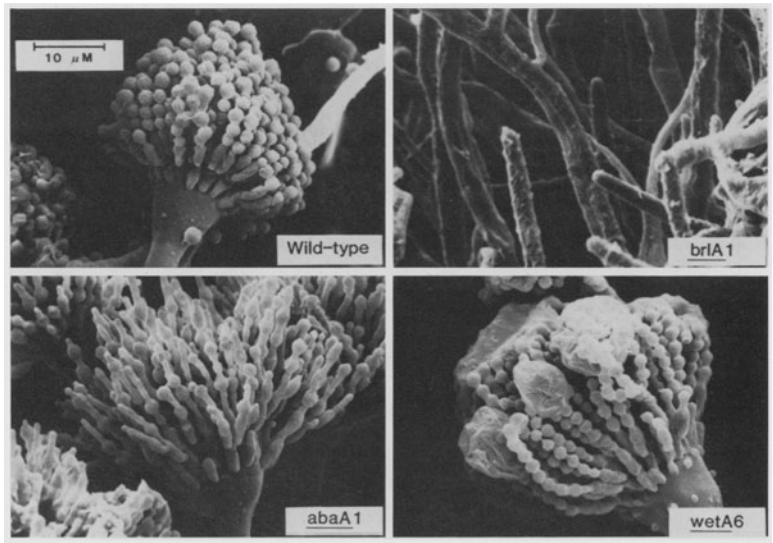
As the two transcripts play distinct roles, their activation is regulated at different levels (Han et al., 1993). While *brlA*α is regulated at the transcriptional level, *brlA*β is regulated at both transcriptional and translational levels. *brlA*α expression requires both *abaA* and *brlA*, while *brlA*β expression is independent of BrlA. In addition, overexpression *brlA*β can induce *brlA*α in the *abaA*$^-$ mutant. The upstream open reading frame of the *brlA*β transcript translationally represses *brlA* expression, thus avoiding premature development before induction. To better understand the transcriptional control of *brlA*α and *brlA*β, Han and Adams (2001) further analyzed the promoters for the two transcripts. They found that two *brlA*β and one *brlA*α sequence fragments function to regulate their expression. In particular, a sequence fragment from the *brlA*β promoter is responsible for *abaA*-mediated repression of *brlA* expression. However, no AbaA binding sites are found in this region, suggesting that the repressive role of *abaA* may be exerted through the negative-feedback regulator VosA (see below).

The BrlA protein is a putative transcription factor (activator) with two C_2H_2 zinc finger motifs at the C terminus (Adams et al., 1988). Disruption of either one of the fingers by mutating the motif cysteines to serines resulted in complete loss of *brlA* activity (Adams et al., 1990). Chang and Timberlake (1992) further verified that *brlA* expression in *Saccharomyces cerevisiae* resulted in *brlA*-dependent activation of *Aspergillus* genes in *S. cerevisiae*. Furthermore, the yeast system was also used to define the proposed consensus sites for BrlA protein binding [BrlA response elements (BREs); 5′-(C/A)(G/A)AGGG(G/A)-3′]. Although the direct binding of BrlA to BREs has not been verified in vitro, a number of developmentally regulated genes, including *abaA*, *wetA*, *rodA*, and *yA* have multiple BREs closely associated with their transcription start sites, which supports that BrlA is a transcription factor.

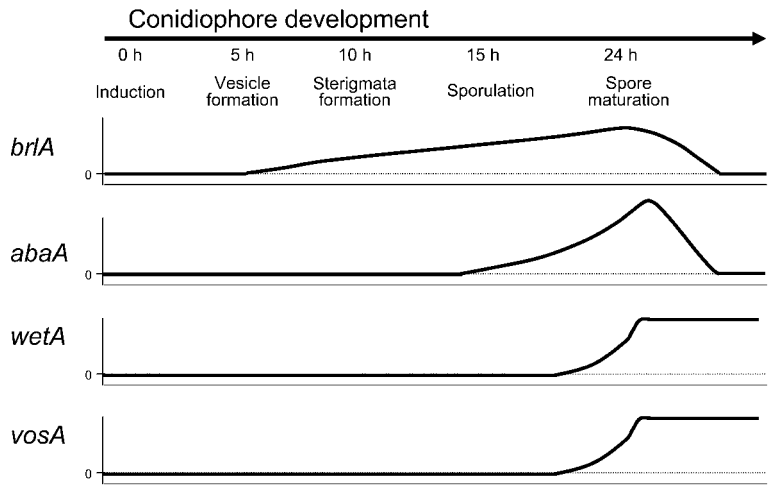
The BrlA protein is localized mainly in vesicles, metulae, and phialides but not in hyphae or mature conidia (Fig. 3B and C), suggesting that its main role is to regulate development and that its presence in hyphae or conidia may have negative effects. Adams et al. (1988) verified this speculation by testing the morphological changes caused by overexpression of *brlA* in vegetative cells and conidia. Overexpression of *brlA* (using the *alcA* promoter) resulted in inhibition of vegetative growth and abnormal differentiation of viable conidia directly from the hyphal tip in submerged culture (Adams et al., 1988; Adams and Timberlake, 1990). The radial growth of the *alcA*(p)::*brlA* strain was also inhibited after being transferred to inducing solid medium. In addition, *alcA*(p)::*brlA* mutant conidia failed to germinate on inducing medium (Adams et al., 1988). Moreover, overexpression of *brlA* activates the expression of *abaA* and *wetA* as well as additional developmentally specific transcripts (Fig. 2). These data further support the conclusion that *brlA* is the key regulator of asexual development and that the misscheduled expression of *brlA* during growth and in mature conidia is undesirable for the fungus.

The functions of BrlA have been studied in other aspergilli. Deletion of the *brlA*-homologous gene eliminates asexual development in *Aspergillus fumigatus* (Mah and Yu,

A



B



C

Gene or class:	*brlA*	*abaA*	*wetA*	*vosA*	Early Genes	Phialide genes	Spore genes
Time of expression	10 h	15 h	24 h	24 h	10 h	15 h	24 h
Sites							
Hyphae	—	—	—	—	—	—	—
Vesicles	+	—	—	—	+	+	+
Metulae	+	+	—	+	+	+	+
Phialides	+	+	+	+	+	+	+
Conidia	—	—	+	+	—	—	+

FIGURE 3 Central regulatory genes' mutants and expression. (A) Phenotypes of *brlA*, *abaA*, and *wetA* mutants (reproduced from Boylan et al., 1987, with permission of the publisher). (B) Expression trends of central regulatory genes during conidiophore development. (C) Time and sites of expression of *Aspergillus* developmental genes. Data are from Boylan et al. (1987), Aguirre et al. (1990), Marshall and Timberlake (1991), and Ni and Yu (2007). The table is adapted and modified from Table 16.1 from Timberlake and Clutterbuck, 1994.

2006) and *Aspergillus oryzae* (Yamada et al., 1999). Yamada et al. (1999) further demonstrated that the misscheduled expression of *brlA* in submerged culture of *A. oryzae* was sufficient to cause the fungus to develop the complex conidiophore structures, including vesicles, phialides, and conidia. These studies clearly demonstrate that BrlA also plays an essential role in controlling conidiophore development in other distantly related *Aspergillus* species.

AbaA Acts as a Genetic Switch To Control Development

Abacus (*abaA*) mutants have nearly normal conidiophores (Fig. 3A), bearing abacus-like structures with swellings at intervals instead of chains of conidia (Clutterbuck, 1969). Morphological examination at the ultrastructural level showed that the metulae of *abaA* mutants produce supernumerary tiers of cells with metula-like rather than phialide-like properties, indicating that *abaA* regulates the differentiation and function of phialides (Sewall et al., 1990b). The *abaA* gene encodes a putative developmental regulator, whose expression is initiated when phialides form at 15 h after induction (Fig. 3B and C) (Boylan et al., 1987). *abaA* mRNA accumulation is dependent on *brlA* activity (Boylan et al., 1987), and BREs have been found in the *abaA* promoter region (Chang and Timberlake, 1992), indicating that *brlA* may directly activate *abaA*. As AbaA is a regulator of development, loss of *abaA* results in the abnormal expression of many developmentally regulated genes (Fig. 2) (Boylan et al., 1987).

Induced activation of *abaA* in vegetative hyphae led to a cessation of growth and accentuated cellular vacuolization, but not conidium formation (Mirabito et al., 1989; Adams and Timberlake, 1990), indicating that overexpression of *abaA* is also undesirable for hyphal growth. Overexpression of *abaA* activates the expression of *wetA* and *brlA*, implying that *abaA* induces *brlA* expression at a certain time during development. However, the genetic interaction between *abaA* and *brlA* seems to be more complex, since *brlA* expression is actually increased in *abaA* null mutants (Aguirre, 1993; Ni and Yu, 2007). As discussed below, a role for *abaA* in repressing *brlA* expression may be exerted through *vosA*.

The AbaA protein contains an ATTS/TEA DNA-binding motif, which is also present in other transcription factors, such as the simian virus 40 enhancer factor TEF-1 and the *S. cerevisiae* Ty1 enhancer binding protein TEC1 (Bürglin, 1991; Andrianopoulos and Timberlake, 1994). AbaA also contains a potential leucine zipper for dimerization (Mirabito et al., 1989). Expression studies involving both *A. nidulans* and *S. cerevisiae* have verified that AbaA can bind to the *cis*-acting regulatory sites identified upstream of the developmentally regulated *yA* gene (Aramayo and Timberlake, 1993). Results from gel mobility shift experiments showed that AbaA binds to the consensus sequence 5′-CATTCY-3′ (ARE), where Y is a pyrimidine (Andrianopoulos and Timberlake, 1994). Multiple AREs are present in the upstream regions of developmentally regulated genes, including *brlAα*, *wetA*, *yA*, *rodA*, and *abaA*. In addition, it was demonstrated that AbaA can bind to the promoter of chitin synthase *chsC*, suggesting that AbaA also regulates chitin biosynthesis during conidiophore development by controlling the transcription level of certain chitin synthase genes (Park et al., 2003).

Homologues of AbaA have been identified in the genomes of *A. fumigatus*, *A. parasiticus*, *A. oryzae*, *A. niger*, *A. terreus*, and *A. clavatus*. Deletion of *abaA* in *A. fumigatus* resulted in phenotypes almost identical to those caused by the *A. nidulans abaA* deletion (S. Gadwal, N. Gao, and J. H. Yu, unpublished data), indicating that, as observed for BrlA, AbaA plays an essential role in conidiation in other aspergilli. In addition, it was found that the *Penicillium marneffei abaA* homologue functions during both conidiation and dimorphic growth phases (Borneman et al., 2000).

WetA Regulates Conidial Wall Formation and Maturation

Wet-white (*wetA*) mutants produce colorless conidia that completely autolyze within a few days, leaving droplets of liquid on the tops of conidial heads (Fig. 3A) (Clutterbuck, 1969). Sewall et al. (1990a) showed that the *wetA* gene is required late in development for the synthesis of a crucial cell wall component, the inner C4 cell wall.

The *wetA* gene was cloned by complementation of a recessive *wet-white* mutant (Boylan et al., 1987). Similar to *brlA*, *wetA* also encodes two transcription units. The larger transcript begins to accumulate at 12 h after induction, and both transcripts are present at very high levels at 24 h after induction. Only the smaller transcript is found in mature conidia (48 h after induction). However, detailed molecular structures for the *wetA* transcripts have not been determined. Similar to that of *brlA*, transcriptional control of *wetA* may be complex and related to developmental control. *abaA*, but not *brlA*, is required for *wetA* expression since overexpression of *abaA* can activate *wetA* in the absence of wild-type *brlA* (Mirabito et al., 1989). In addition, *wetA* is self-regulated, as it is not expressed in *wetA* temperature-sensitive mutants (Boylan et al., 1987; Mirabito et al., 1989).

The *wetA* gene encodes a 60-kDa protein that is rich in serine (14%), threonine (7%), and proline (10%) (Marshall and Timberlake, 1991). Although no known DNA binding domains have been identified in the WetA protein, the *wetA* gene has been proposed to encode a regulator of spore-specific gene expression (Marshall and Timberlake, 1991). This hypothesis is based on the finding that *wetA* alone is sufficient to activate many sporulation-specific genes (Marshall and Timberlake, 1991) and *wetA* mutants do not accumulate many sporulation-specific mRNAs (Boylan et al., 1987). Furthermore, overexpression of *wetA* in vegetative cells inhibits hyphal growth and results in excessive branching and activation of spore-specific genes (Marshall and Timberlake, 1991). Overexpression of *wetA* does not result in *brlA* or *abaA* activation and never leads to premature conidiation. Taken together, these results suggest that *wetA* is responsible for activating a set of genes required for spore formation and maturation, and that may function in forming the final two conidial wall layers or directing their assembly.

WetA homologues have been found in *A. fumigatus*, *A. parasiticus*, *A. oryzae*, *A. niger*, *A. terreus*, and *A. clavatus*. Disruption of *wetA* in *A. fumigatus* resulted in spore defects almost identical to those caused by the *A. nidulans wetA* deletion (Gadwal et al., unpublished), indicating that essential functions for BrlA, AbaA, and WetA in conidiation are conserved in aspergilli. Moreover, the *Penicillium chrysogenum wetA* homologue (*PwetA*) fully complemented the *wetA* deletion in *A. nidulans*, suggesting that the mechanisms controlling sporulation are evolutionarily conserved in *Aspergillus* and *Penicillium* (Prade and Timberlake, 1994).

Developmental Modifiers StuA and MedA

Two other developmental modifiers, *stuA* and *medA*, are necessary for the precise spatial pattern in the multicellular conidiophore (Miller et al., 1991, 1992). While mutations

in either gene caused abnormal conidiophores, both *stuA* and *medA* mutants were able to produce some viable conidia, and they have been termed oligosporogenous mutants (Clutterbuck, 1969).

Stunted (*stuA*) mutants produce greatly shortened conidiophores and lack normal metulae and phialides (Clutterbuck, 1969; Miller et al., 1991). Abnormal pigmented spores with normal viability differentiate directly from buds formed on the conidiophore vesicles. In addition, *stunted* mutants are sterile, failing to produce Hülle cells and cleistothecia. The pleiotropic effects of *stunted* mutants indicate that there are important overlapping regulatory mechanisms between asexual and sexual development.

Like the *brlA* and *wetA* loci, *stuA* encodes two overlapping transcripts, *stuA*α and *stuA*β, (Miller, et al., 1992). *stuA*α initiates within the first intron found in *stuA*β, and both transcripts have long 5′ untranslated regions and multiple mini-ORFs. These mini-ORFs play an important role in the translational control of *stuA*, and their deletion differentially affects temporal development and morphogenesis during both asexual and sexual development (Wu and Miller, 1997). *stuA* is expressed during vegetative growth, levels are increased 50 times following the acquisition of developmental competence, and an additional 15-fold increase is observed following developmental induction (Miller et al., 1992). Enhanced *stuA* expression is cell specific and requires a wild-type copy of the *brlA* gene (Miller et al., 1992).

The *stuA* gene encodes a 590-amino-acid (aa) protein containing a basic helix-loop-helix DNA binding domain, which is also found in several other proteins (including ASM-1, PHD-1, StuA, EFGTF-1, and SOK2) and has therefore been named APSES (Aramayo et al., 1996; Dutton et al., 1997). A protein in which the bipartite nuclear localization signal of *stuA* is fused to green fluorescent protein is localized in the nucleus (Suelmann et al., 1997). This result, in conjunction with the evidence that ASM-1 is localized to the nucleus (Aramayo et al, 1996), suggests that StuA may also be found in the nucleus and function as a transcription factor. Further in vitro analysis verified that StuA bound to MCB-like motifs and promoted transcription from the MCB motif in yeast (Dutton et al., 1997). The StuA response element [5′-(A/T)CGCG(T/A)N (A/C)-3′] is found upstream of developmental genes such as *brlA*α and *abaA* and cell cycle genes, including *nimE* and *nimO*. Supporting StuA as a potential regulator of *brlA* and *abaA*, mutations in *stuA* lead to mislocalized expression of *abaA*(p)::*lacZ* and *brlA*(p)::*lacZ* fusions, although temporal expression of these two genes is normal in *stuA* mutants (Miller et al., 1992; Aguirre, 1993). Despite the activating capacity of StuA in yeast, it represses transcription of *abaA* in *A. nidulans* (Dutton et al., 1997).

Medusa (*medA*) mutants produce abnormal conidiophores with multiple layers of sterigmata, from which conidia are formed normally (Clutterbuck, 1969). Occasionally, the mutant conidiophores bear secondary conidiophores. The mutants fail to produce cleistothecia, although they have normal Hülle cells. Like the regulatory genes *brlA*, *wetA*, and *stuA*, the *medA* gene encodes two transcription units (Miller, 1993). Both transcripts have long 5′ untranslated regions that contain multiple mini-ORFs. This again suggests the possibility of translational regulation. *medA* is expressed at high levels during vegetative growth, and its expression declines during developmental induction (Miller, 1993).

The *medA* gene regulates the expression of *brlA* and *abaA* (Busby et al., 1996). *medA* represses premature expression of both *brlA*α and *brlA*β during early development. In addition, *brlA*β is downregulated during later stages of development. Therefore, in *medA* mutant strains, the ratio of *brlA*α and *brlA*β is lower than in wild-type strains. On the other hand, *medA* is required for expression of *abaA* during development. Interestingly, an extra copy of *brlA* can suppress *medA* mutations and restores *abaA* expression to normal level in a cold-sensitive manner (Busby et al., 1996), suggesting that MedA and BrlA may form a heterodimeric protein complex to regulate other genes. In contrast, the presence of an extra copy of *stuA* in *medA* mutant enhances the *medusoid* phenotype, causes the total absence of conidia, and represses the expression of *brlA*α and *abaA* (Busby et al., 1996), indicating that altered gene dosage ratios of *brlA*, *medA*, and *stuA* change conidiophore development. In general, a low *stuA*-to-*brlA* ratio activates conidiation, while a high ratio inhibits conidiation and results in the proliferation of unicellular sterigmata.

StuA homologues have been identified in *A. fumigatus*, *A. parasiticus*, *A. oryzae*, *A. niger*, *A. terreus*, and *A. clavatus*. MedA homologues are also found in these fungi, with the exception of *A. niger*. Afs*tuA* in *A. fumigatus* (Sheppard et al., 2005), Fos*tuA* in *Fusarium oxysporum* (Ohara and Tsuge, 2004), Pms*tuA* in *Penicillium marneffei* (Borneman et al., 2002), Ren1 (MedA homologue) in *Fusarium oxysporum* (Ohara et al., 2004), and ACR1 (MedA homologue) in *Magnaporthe grisea* (Nishimura et al., 2000) all play a regulatory role in controlling conidiation.

Sporulation-Specific Genes

Sporulation-specific genes have been divided into four classes (classes A through D), based on the effects of *brlA*, *abaA*, or *wetA* mutations on their expression during conidiation (Boylan et al., 1987; Adams et al., 1988; Mirabito et al., 1989). Class A genes, such as *yA* and *rodA*, are induced by either *brlA*, *abaA*, or both and are independent of *wetA* (Fig. 2). These genes are expected to be involved in early developmental events (Fig. 3C). Class B genes, including *wA*, are activated by *wetA*, independent of *brlA* and *abaA*. Class B genes are predicted to encode spore-specific functions (Fig. 3C). Class C and D genes require combined activities of the three central regulatory genes for their expression and have been proposed to encode phialide-specific functions (Fig. 3C) (reviewed by Adams et al., 1998). Class C and D genes are distinguished from one another by their expression patterns during normally induced development in wild-type and mutant strains.

VosA Completes Conidiation

A recent study has identified the novel regulator VosA as a master regulator of conidia maturation and completion of development in *A. nidulans* (Ni and Yu, 2007). The *vosA* gene is expressed specifically during the formation of both sexual and asexual spores. Moreover, deletion of *vosA* results in the lack of trehalose in spores, a rapid loss of the cytoplasm, cellular organelles, and spore viability (Fig. 4), and a dramatic reduction in tolerance of conidia to heat and oxidative stresses. Furthermore, VosA is localized mainly in the nucleus of mature conidia and it contains a potential transcription activation domain at the C terminus and a potential DNA binding domain at the N terminus (Ni and Yu, 2007; Ni, 2008). Taken together, these findings indicate that VosA is a transcription factor that primarily controls the later stages of sporulation, including trehalose biogenesis. The VosA protein first accumulates in metulae and phialides and later is localized in the nucleus of conidia, where it plays two main roles: (i) activating spore maturation-related genes and (ii) conferring negative-feedback regulation of development-specific genes through repressed

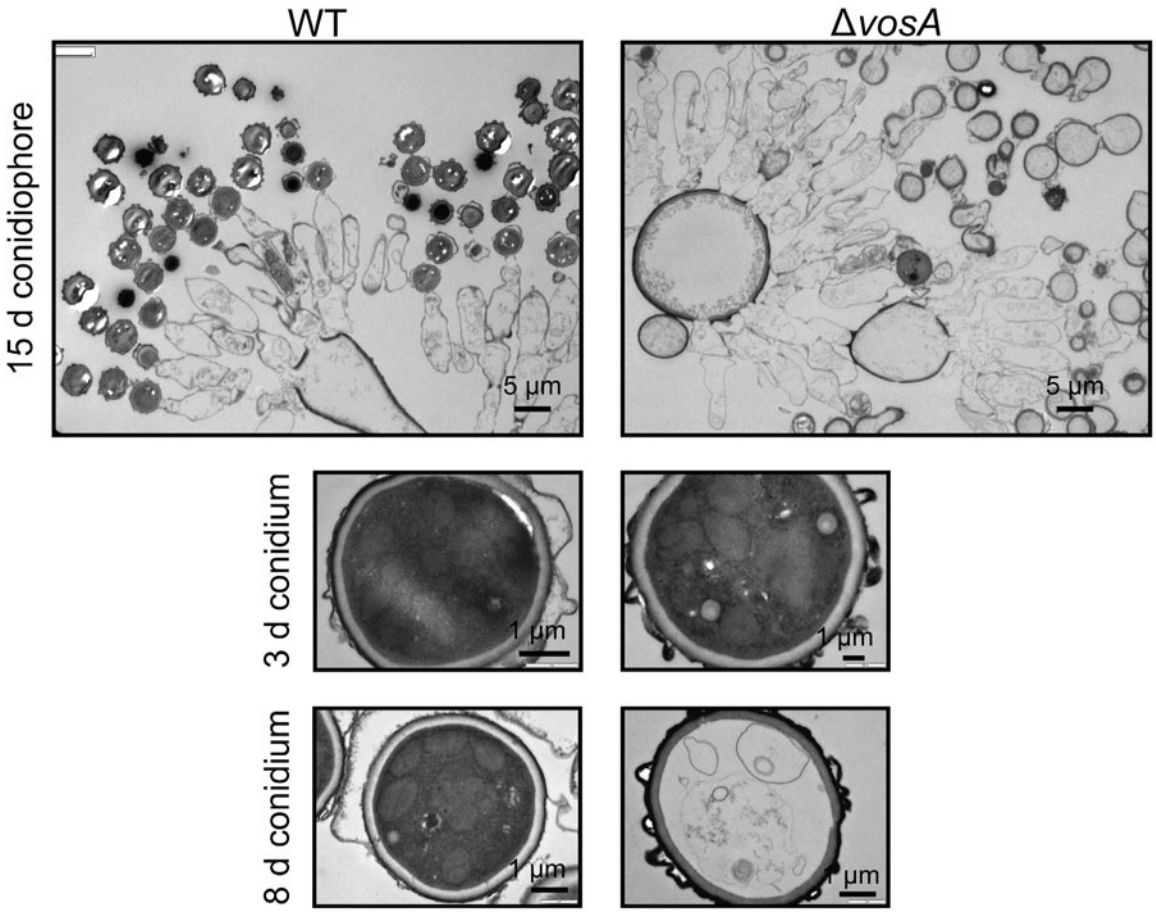


FIGURE 4 Requirement of *vosA* in integrity of spores. Transmission electron microscopic analysis of 15-day-old conidiophores and 3- and 8-day-old conidia of wild-type (WT) and Δ*vosA* strains (adapted and modified from Ni and Yu, 2007). The deletion of *vosA* caused loss of cytoplasm and organelles including the nucleus in old conidia.

expression of *brlA* (Fig. 2). The identification of VosA clearly divides conidiophore formation and conidial maturation into two distinct phases at the genetic level.

UPSTREAM ACTIVATORS OF CONIDIATION

As mentioned above, *brlA* is the key activator of conidiation in *A. nidulans* (Adams et al., 1988; Chang and Timberlake, 1992). Six genes (*fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*) that are required for the proper expression of *brlA* have been identified. Mutations in any of these genes result in "fluffy" colonies that are characterized by proliferation of undifferentiated cotton-like masses of vegetative cells (Fig. 5) (reviewed by Adams et al., 1998). The possible interactions between various *flb* genes were assessed by the phenotypes of different combinations of double mutants (Wieser et al.,

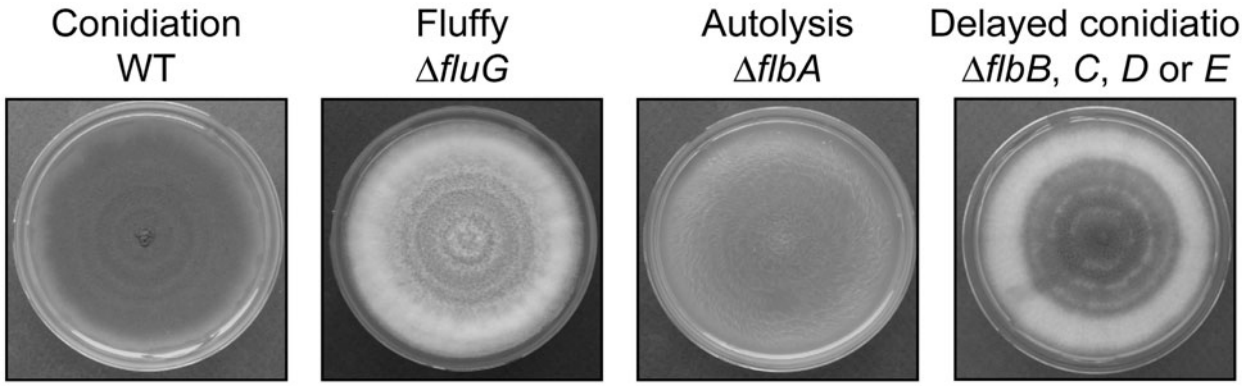


FIGURE 5 Phenotypes of fluffy mutants.

1994) and by testing the genetic requirements for the conidiophore formation observed following overexpression of *flu*G, *flb*A, or *flbD* (Wieser and Adams, 1995; Lee and Adams, 1996). This resulted in the proposed gene order *flu*G →→*flbE*→*flbD*→*flbB*. As *flu*G functions first in this regulatory network, *flu*G overexpression requires the activities of *flb*C, *flb*A, and *flbD* to activate *brl*A and conidiation in submerged culture. Identification and characterization of these developmental activators greatly enhanced our understanding of the molecular mechanisms for upstream regulation of conidiation in *Aspergillus*.

FluG-Dependent Initiation of Conidiation

The *flu*G gene encodes a cytoplasmically localized protein that is present at relatively constant levels throughout the life cycle. Loss of *flu*G function results in the absence of both conidiation and production of sterigmatocystin (ST), the penultimate precursor of the better-known potent carcinogen aflatoxin (Lee and Adams, 1994a; Hicks et al., 1997; Yu and Keller, 2005). The C-terminal half of FluG contains a glutamine synthetase I-like domain (Lee and Adams, 1994a), and the N-terminal half is similar to the early nodulin gene product MtN6 in *Medicago truncatula* (Mathis et al., 1999). Interestingly, the N-terminal half of FluG is dispensable for conidiation (D'Souza et al., 2001), indicating that development-specific function resides in the C-terminal half. Corroborating this idea, overexpression of full-length FluG or the C terminus in vegetative cells was sufficient to cause activation of *brl*A and development of conidiophores in liquid submerged culture, suggesting a direct positive role for the C-terminal region of FluG in regulating conidiation (Lee and Adams, 1996; reviewed by Adams et al., 1998; and D'Souza et al., 2001).

The fact that conidiation in *flu*G mutants can be rescued by their being grown in the proximity of wild-type strains or other developmental mutants led to the early hypothesis that FluG is involved in the constitutive synthesis of an extracellular sporulation-inducing factor that might be related to glutamine or glutamate (reviewed by Adams et al., 1998). According to this theory, FluG activity was thought to result in (i) activation of development-specific functions that require the products of other genes including *flbB*, *flb*C, *flbD*, *flbE*, and *brl*A (Adams et al., 1988; Wieser and Adams, 1995; Lee and Adams, 1996); and (ii) activation of FlbA (see below), which then inactivates FadA signaling of proliferation (Yu et al., 1996). Both of these processes must occur if development is to proceed.

The molecular events responding to FluG activity were partially revealed by analysis of suppressor mutations (suppressor of *flu*G [*sfg*]) that bypass the need for *flu*G in conidiation and ST production (Seo et al., 2003, 2006). Seo et al. have proposed that the *flu*G-dependent initiation of asexual sporulation is independent of, and parallel to, G-protein-mediated growth signaling, primarily due to the inability of mutations in FadA or SfaD to suppress Δ*flu*G (Yu et al., 1996; Rosén et al., 1999). It was hypothesized that identification of these suppressors of *flu*G would provide specific components that function downstream of FluG in the asexual sporulation pathway. Identification and characterization of the key suppressor SfgA led to a new working model that SfgA directly/indirectly represses asexual development and FluG activity is required to remove the intracellular repressive effects imposed by SfgA:

FluG ⊣ [SfgA→ repressors] ⊣ other Flb genes → BrlA → sporulation (see below).

Mah and Yu (2006) examined the functions of Af*flu*G in *A. fumigatus*. As in *A. nidulans*, levels of the Af*flu*G transcript are relatively constant throughout the life cycle. The Af*flu*G deletion mutant sporulates normally on solid medium, indicating that activation of *A. fumigatus* conidiation in the presence of air does not require the activity of FluG. However, the Af*flu*G deletion mutant did not produce conidiophores in submerged liquid culture, whereas *A. fumigatus* wild-type strains sporulate abundantly after 24 h under these conditions. Moreover, the Af*flu*G deletion mutant showed reduced conidiation levels and delayed expression of Af*brl*A under the synchronized developmental induction conditions. Mah and Yu (2006) concluded that while the presence of air can bypass the need for AfFluG in conidiophore development, AfFluG plays a positive role during *A. fumigatus* conidiation and in expression of Af*brl*A. These findings led to the hypothesis that *A. fumigatus* has multiple pathways activating expression of Af*brl*A.

FlbA Balances Growth and Development

Loss-of-function *flb*A mutants are distinguished from other mutants by the fact that they begin as a fluffy nonconidial colony but by 3 days after inoculation, the center of the colony begins to disintegrate and by 5 days postinoculation the entire colony has autolyzed (Fig. 5) (Lee and Adams, 1994b; Wieser et al., 1994). The importance of *flb*A in controlling *brl*A expression was made clear by the observation that overexpression of *flb*A in vegetative cells led to *brl*A activation and conidiation (Lee and Adams, 1994b). The predicted *flb*A product is related to *S. cerevisiae* Sst2p, which regulates the activity of the G-protein-mediated signal transduction pathway controlling yeast pheromone response and mating (Dietzel and Kurjan, 1987). The similarity observed between FlbA and Sst2p led to the initial proposal that FlbA functions to regulate the activity of a signal transduction pathway during the response to the putative *flu*G signal for conidiation. Later, it was found that FlbA belongs to a group of proteins called regulator of G protein signaling (RGS) and that FlbA is necessary for the proper control (attenuation) of vegetative growth signaling mediated by a heterotrimeric G protein (Yu et al., 1996; Rosén et al., 1999; Seo et al., 2005; Seo and Yu, 2006). Activities of FlbA are required for coordinating vegetative growth and fungal development (see below).

Potential Upstream Transcription Factors for Conidiation

Mutational inactivation of *flbB*, *flb*C, *flbD*, or *flbE* results in a third, distinct class of developmental defects classified as delayed conidiation (Fig. 5) (Wieser et al., 1994). This phenotype differs from the fluffy phenotype observed for *flu*G and *flb*A loss-of-function mutants in that delayed conidiation mutants have the ability to produce conidiophores in the center of the colonies after extended incubation while the colony margin remains fluffy. These genes have been cloned by complementation of recessive mutations (Wieser and Adams, 1995; Wieser, 1997; Etxebeste et al., 2008).

The *flbD* gene is predicted to encode a 308-aa polypeptide with high identity in its N terminus to the DNA binding domain found in a group of transcription factors that includes the human proto-oncogene c-*myb* (Wieser and Adams, 1995). Overexpression of *flbD* in submerged culture led to the formation of complete conidiophores composed of stalks, vesicles, sterigmata, and viable conidia. This contrasts with development observed following forced expression of *brl*A or *flb*A, which results in the elaboration of abnormal conidiophores with conidia forming directly from hyphal tips (Adams et al., 1988; Lee and Adams, 1994b). Because *flbD* mRNA is present at relatively constant levels in vegetative hyphae and after development

initiation in wild-type cells, Wieser and Adams (1995) proposed that the activity of the putative FlbD DNA binding protein is regulated posttranscriptionally in response to early sporulation signals, resulting in the transcriptional activation of other developmental regulators, such as *brlA*.

The *flb*C gene is predicted to encode a C_2H_2 zinc finger DNA binding protein (Wieser, 1997). FlbC and FlbD apparently contribute independently to developmental activation, because mutations in both *flb*C and *flbD* have an additive effect on development and *flbD* overexpression causes development in the absence of *flb*C (Wieser and Adams, 1995). The *flbE* gene is predicted to encode a novel protein (~200 aa) that might function as a transcriptional activator necessary for normal conidiation in *Aspergillus* species (N. J. Kwon, K. S. Shin, and J. H. Yu, unpublished data).

The *flbB* gene encodes a potential transcriptional activator with a basic leucine zipper (b-ZIP) motif (Vinson et al., 1989) restricted to filamentous fungi (Etxebeste et al., 2008). FlbB accumulates at the hyphal tips during early vegetative growth and then localizes in the apical nuclei, suggesting that a specific mechanism may activate FlbB and trigger its nuclear import. The *flbB* mRNA begins to accumulate during vegetative growth and remains at high levels until early phases of asexual development. A reduction of the *flbB* mRNA levels coincides with the initiation of *brlA* expression. Accumulation of *flbB* mRNA resumes 12 h after asexual developmental induction and *flbB* mRNA is present in conidia and ascospores (sexual spores). Coupled with the observations that the deletion or misscheduled overexpression of *flbB* results in impaired conidiation, these observations suggest that tight regulation of timing and levels of FlbB expression is crucial for developmental progression. Importantly, Etxebeste et al. (2008) showed that FlbB activation is associated with the production of a second diffusible conidiation signal, acting downstream from the FluG factor.

NEGATIVE REGULATION OF CONIDIATION

FluG-Dependent Asexual Development in *A. nidulans* Occurs via Derepression of *sfgA*

As mentioned above, the first negative regulator of conidiation in *A. nidulans*, SfgA (suppressor of *flu*G), was identified during analysis of *flu*G suppressors (Seo et al., 2003, 2006). To investigate the molecular events responding to FluG activity, Seo et al. (2003) isolated extragenic suppressor mutations that restore a wild-type developmental phenotype to a mutant *flu*G strain. They treated a Δ*flu*G haploid strain with a chemical mutagen and identified four suppressor genes: *sfg*A, *sfg*B, *sfg*C, and *sfgD*. The major isolates (~80%) were mapped to *sfg*A, indicating that SfgA may play a central role in regulating downstream of FluG.

The *sfgA* gene encodes a novel 601-residue protein with a Gal4-type $Zn(II)_2Cys_6$ binuclear DNA-binding motif and a nuclear localization signal close to the N terminus, suggesting that SfgA may function as a transcription factor, likely an activator (Seo et al., 2006). Deletion of *sfgA* bypassed the need for *flu*G during conidiation and sterigmatocystin production, indicating that the primary role of FluG is to remove the repressive effects imposed by SfgA. Consistent with the proposed regulatory role of SfgA, deletion of *sfgA* led to inappropriate conidiophore development in submerged culture. Moreover, overexpression of *sfgA* inhibited conidiation (Fig. 6A) (Seo et al., 2006), further supporting the idea that SfgA functions as a negative regulator of conidiation. To determine the genetic position of SfgA, Seo et al. (2006) carried out a series of double-mutant analyses. Deletion of *sfgA* could not bypass the need for *flbA*, *flbD*, *flbB*, *flb*C, or *brlA* during conidiation, indicating that *sfgA* functions upstream of these genes. In contrast, Δ*sfgA* partially suppressed Δ*flbE*, demonstrating that SfgA functions downstream or at the same level as FlbE. Based on the data, the authors proposed a working model in which FlbE and SfgA function at the same level, SfgA activates downstream target genes designated SARs (SfgA-activated repressors of conidiation), and FlbE competes with SfgA to inhibit the activities of SARs (Seo et al. 2006).

A Gain-of-Function Screen Identifies Additional Repressors of Conidiation

The findings from SfgA study indicate that SfgA may activate a group of repressors of conidiation and overexpression of these repressors may inhibit conidiation. In an effort to identify additional repressors of conidiation, Ni and Yu (2007) carried out a gain-of-function genetic screen. They transformed a wild-type strain with the pRG3-AMA1-based wild-type library, resulting in transformants carrying multiple copies of various genes. Using a visual screen for transformants with a fluffy phenotype, they identified three genes: AN1959 (VosA), AN6578 (OsaA), and AN6437. All three proteins are conserved in filamentous fungi (Ni, 2008). Deletion of AN6437 caused no apparent phenotypes (Ni, 2008). OsaA, containing the Tos9 domain at the N terminus, is a homologue of *Candida albicans* Wor1, which is a putative transcription factor regulating the white-opaque phase transition (Zordan et al., 2006, 2007). Deletion of *osaA* resulted in colonies with mixed sectors of mainly asexual or sexual development. This suggests that OsaA may also play regulatory roles during the asexual and sexual developmental phase transitions (Ni, 2008).

VosA Represses Conidiation and Exerts Feedback Regulation of Development

As described above, VosA couples the formation of spores and focal trehalose biogenesis and functions as a master regulator of spore maturation in *A. nidulans*. During the late phase of sporulation, VosA functions in activating genes required for the maturation process but represses certain development-specific genes. Supporting its repressive role in controlling conidiation, Ni and Yu (2007) found that the absence of *vosA* leads to uncontrolled activation of asexual development, whereas enhanced expression of *vosA* blocks sporulation (Fig. 6B).

At the molecular level, deletion of *vosA* results in nearly constitutive accumulation of *brlA* (and *rodA* [Stringer et al., 1991]) mRNA even at 48 h after developmental induction, and in conidia. Conversely, levels of *wetA* (and *wA* [Mayorga and Timberlake, 1992]) mRNA are considerably lower in the Δ*vosA* mutant than in the wild type. However, deletion of *vosA* causes a minute increase in the expression of *abaA* (and *yA* [Aramayo and Timberlake, 1990]). Moreover, overexpression of *vosA* inhibits accumulation of *brlA* mRNA. Collectively, these results indicate that VosA represses *brlA* expression but activates *wetA*, suggesting that VosA is not a simple upstream or downstream regulator of central regulatory genes. In other words, VosA plays a complex regulatory role in coordinating and balancing the expression of central regulatory genes. As summarized in Fig. 2, expression analyses reveal that both *abaA* and *wetA* are required for *vosA* mRNA accumulation and *vosA* and *wetA* activate each other (Fig. 2) (Ni and Yu, 2007). In addition, *abaA* and *wetA* are necessary for the repression of *brlA*, and the repressive roles may be primarily attributed to

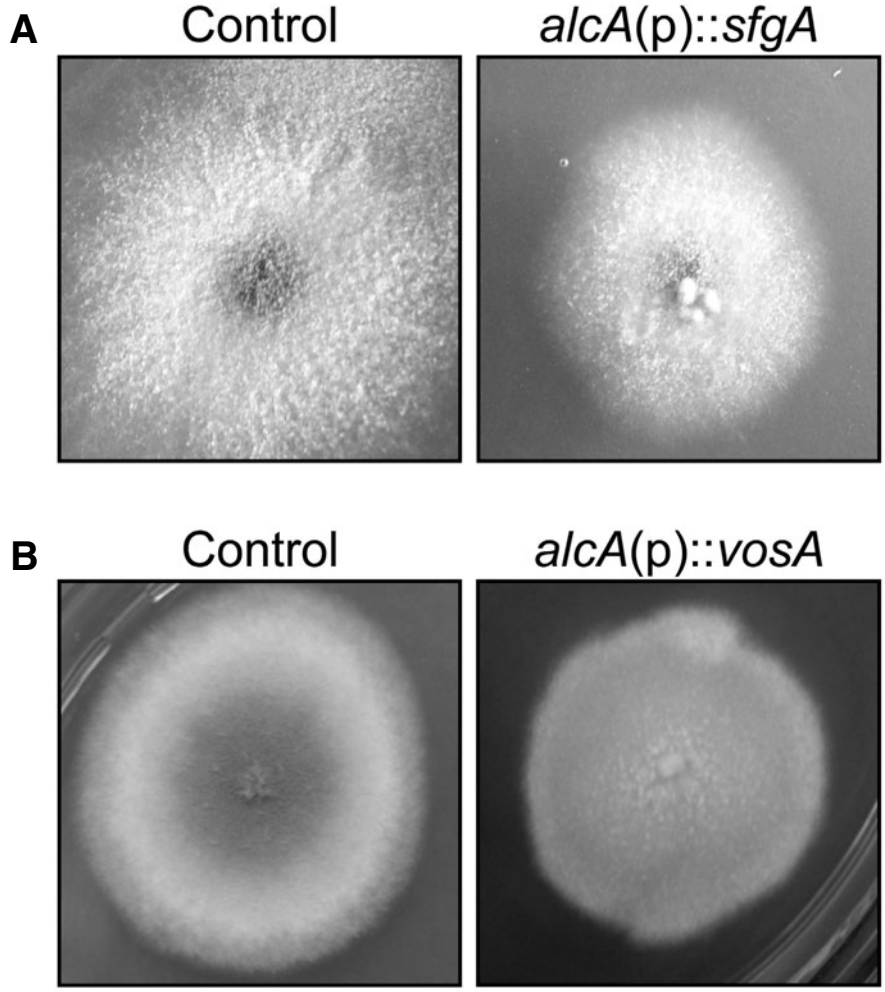


FIGURE 6 Overexpression of *sfgA* or *vosA* inhibits conidiation. (A) Overexpression of *sfgA* [*alcA*(p)::*sfgA*] colony exhibits white undifferentiated hyphae (fluffy) with low levels of conidiation in the center. (B) Overexpression of *vosA* [*alcA*(p)::*vosA*] colony exhibits complete block of conidiation (reproduced from Ni and Yu, 2007, with permission).

VosA functioning in a negative-feedback loop. Moreover, both *brlA* and *wetA* are subject to autoactivation and *wetA* functions to negatively regulate *abaA*. Results from Northern blot analysis suggest that *brlA* and *wetA* are potential direct targets of VosA and that VosA may be the direct target of AbaA. Supporting this idea, two AbaA binding sites are found in the promoter of *vosA* (Ni, 2008).å

As described above, VosA is a potential transcription factor that may control the late process of sporulation, including trehalose biogenesis. Chromatin immunoprecipitation-microarray (ChIP-chip) analyses have shown that VosA binds to more than 2,000 genes, including *vosA*, *brlA*, *wetA*, *tpsA*, *tpsC*, *orlA*, and *treA* (Ni, 2008), indicating that VosA may affect a great number of genes.

In summary, two negative regulators, SfgA and VosA, have been identified and characterized. SfgA functions upstream to repress the roles of FluG, while VosA exerts feedback regulation of *brlA* expression in a downstream pathway (Fig. 2). Since VosA and SfgA are highly conserved in *Aspergillus* species, the identification and characterization of their targets and homologues in other fungi should reveal new information regarding conidiation in *Aspergillus*.

VELVET PROTEINS, REGULATORS OF FUNGAL DEVELOPMENT

Light-Dependent Roles for VeA in Controlling Fungal Development

Besides the genetic control of conidiation mentioned above, environmental factors, including light, pH, and growth sources, also influence asexual development (Mooney and Yager, 1990; Skromne et al., 1995; Peñalva and Arst, 2004). In *A. nidulans*, light is necessary for inducing asexual sporulation, while in darkness the fungus undergoes sexual development to form the sexual fruiting bodies cleistothecia (Mooney and Yager, 1990; Yager, 1992). This property relies on the presence of the wild-type allele of the *velvet* gene (*veA*). The *veA1* allele, which has a point mutation in the first ATG and instead uses M37 as the start codon, abolishes the light dependence of conidiation.

Laboratory strains carrying the *veA1* allele show increased conidiation and reduced sexual development under both light and dark conditions, i.e., the *velvet* phenotype. In addition, in *A. nidulans* the *veA* deletion results in the complete loss of cleistothecium formation (Kim et al., 2002; Kato et al., 2003) and increased conidial production (Kim et al., 2002). These results suggest that VeA functions during activation of sexual development and repression of conidiation.

The *velvet* Complex Bridges Fungal Development and Secondary Metabolism

Two recent studies have verified that the N-terminal region (36 aa) of VeA plays important functions in controlling light sensing and sexual development (Stinnett et al., 2007; Bayram et al., 2008a). Stinnett et al. showed that one putative nuclear localization signal motif from amino acids K28 to R44 of the VeA protein, truncated in VeA1, is necessary for nuclear localization of VeA. VeA is located mainly in nuclei in the dark but is abundant in the cytoplasm under light. However, the VeA1 mutant protein is found predominantly in the cytoplasm, independent of illumination. This finding suggests an important role for the VeA N-terminal region in balancing sexual and asexual development. Another study revealed an additional role for this N-terminal fragment: binding to VelB (Bayram et al., 2008a). VelB was identified as a protein that interacts with VeA by tandem affinity purification. Since it was shown to have sequence similarity to VeA at the N terminus, this protein was named velvet-like protein B (VelB) (Ni and Yu, 2007).

velB deletion mutants, similar to Δ*veA* strains, display a light-dependent developmental pattern and fail to form sexual fruiting bodies under any conditions. Neither overexpression of *velB* nor *veA* could rescue defects caused by deletion of the other gene. These data (Bayram et al., 2008a) suggest that these two proteins are functionally dependent on one other in controlling sexual development. Although VelB cannot bind to VeA1 (with a truncated N-terminal region), *veA1* laboratory strains can still undergo sexual development under certain conditions. This suggests that the presence of both VelB and VeA (or VeA1), but not VeA-VelB binding, is required for sexual development.

Deletion of *velB* resulted in less conidial production, while overexpression of *velB* led to a twofold increase in the yield of conidia but a normal amount of cleistothecia, indicating that VelB also plays roles in controlling conidiation (Bayram et al., 2008a). Although the *veA* deletion mutant (unlike Δ*velB)* is not affected in asexual development, a double mutant lacking both *veA* and *velB* exhibits the fluffy phenotype in the dark and produces fewer conidia in the light, suggesting that VeA and VelB function redundantly in modulating asexual development (Ni, 2008).

In summary, VeA and VelB function interdependently to regulate sexual development but play redundant roles in controlling asexual development, suggesting that complex mechanisms exist for the regulation of both asexual and sexual development.

In addition to their roles in controlling fungal development, VeA and VelB also regulate secondary metabolism by interacting with LaeA (Fig. 7) (Bayram et al., 2008a). LaeA has been identified as a global regulator of secondary mechanism (Bok and Keller, 2004). Without LaeA function, the expression of metabolic gene clusters, including the ST (a carcinogen), penicillin (an antibiotic), and lovastatin (an antihypercholesterolemic agent) gene clusters,

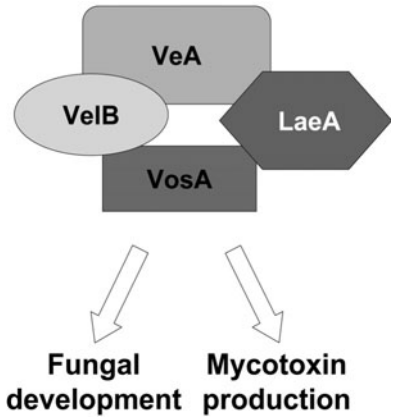


FIGURE 7 The VosA/VelB/VeA/LaeA complex coordinates fungal development and mycotoxin production.

are blocked (Bok and Keller, 2004). In contrast, *laeA* overexpression leads to increased penicillin and lovastatin gene transcription and related product formation. Multiple studies have also shown that *veA* is essential for normal production of mycotoxin and other secondary metabolites in many *Aspergillus* species (Kato et al., 2003; Calvo et al., 2004; Duran et al., 2007). In *A. nidulans*, deletion of *veA* or *laeA* results in loss of ST production, while deletion of *velB* decreases the yield of ST (Bayram et al., 2008a). Since VeA directly binds to VelB through its N terminus and to LaeA through its C terminus (Fig. 7), the roles of VeA and VelB in controlling mycotoxin production may be mediated through LaeA function. More interestingly, VosA has been shown to interact with VelB and LaeA in VelB tandem affinity purification tagging and yeast two-hybrid experiments (Fig. 7) (Bayram et al., 2008a). Δ*vosA*;*veA*$^{+}$ strains produce normal amounts of ST, while the Δ*vosA*;*veA1* strains cannot produce ST (Ni, 2008). These data suggest that the VelB-VeA-LaeA or VelB-VosA-LaeA protein complex is required for mycotoxin production. The Δ*vosA*;*veA1* strain only contains the VeA1-LaeA protein complex, which cannot induce ST production. The detailed molecular mechanism regarding how these protein complexes regulate fungal development and secondary metabolism is still unknown.

The *velvet* Proteins Are Conserved in Filamentous Fungi

The *velvet* homologues, including VeA, VosA, VelB, and VelC, have defined a new protein family. A series of alignment studies have shown that these four proteins have a highly conserved N-terminal domain (Ni and Yu, 2007) and that they are found in almost all ascomycetes and basidiomycetes (Fig. 8). VeA homologues have been reported to control fungal development and secondary metabolism in multiple fungal species. They (i) regulate conidiation, sclerotial production, and mycotoxin production in *A. parasiticus* (Calvo et al., 2004); (ii) regulate conidiation in a nitrate-dependent manner in *A. fumigatus* (Krappmann et al., 2005); (iii) alter the expression of genes associated with aflatoxin and sclerotial production in *A. flavus* (Cary et al., 2007); (iv) influence cell wall integrity, hyphal growth, and morphological development in *Fusarium verticillioides* (Li et al., 2006); (v) modulate conidiation in *Neurospora crassa* (Bayram et al., 2008b); and (vi) regulate antibiotic

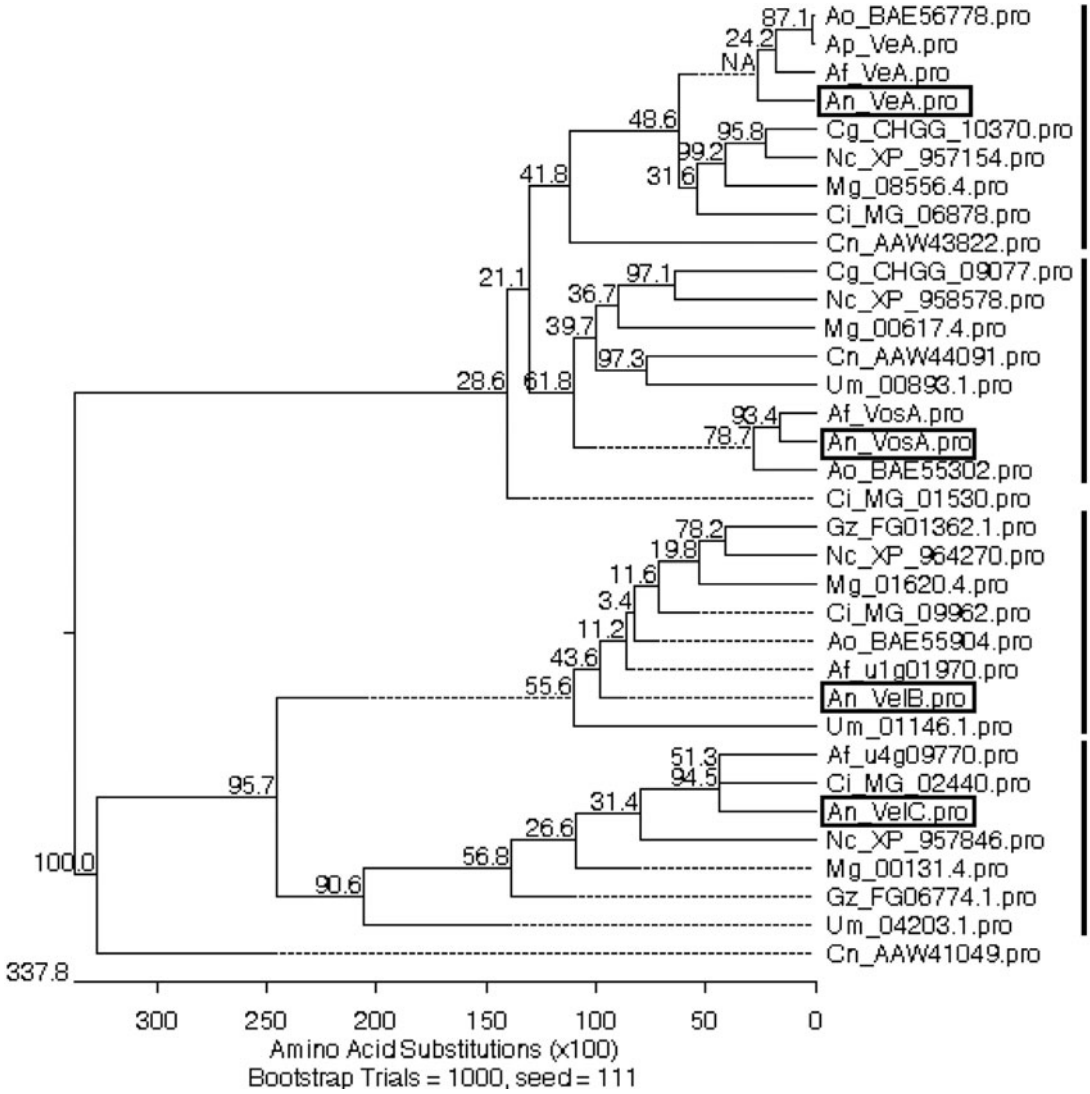


FIGURE 8 A phylogenetic tree of proteins similar to VosA (reproduced from Ni and Yu, 2007, with permission). The ClustalV method was used for protein alignment (Chenna et al., 2003), and the phylogenetic tree was generated by MegAlign in Lasergene v7.0 (DNASTAR). Abbreviations: An, *A. nidulans*; Af, *A. fumigatus*; Ao, *A. oryzae*; Ap, *A. parasiticus*; Ci, *Coccidioides immitis*; Mg, *M. grisea*; Cg, *Chaetomium globosum*; Nc, *N. crassa*; Um, *Ustilago maydis*; Cn, *Cryptococcus neoformans*; Gz, *Gibberella zeae*.

cephalosporin C biosynthesis and hyphal fragmentation in *Acremonium chrysogenum* (Dreyer et al., 2007). As mentioned above, VosA has been reported as a master regulator of spore maturation and regulator of trehalose biogenesis in *A. nidulans*, and its roles seem to be conserved in *A. fumigatus* (Ni and Yu, 2007). As mutational inactivation of *vosA*, *veA*, or *velB* caused pleiotropic effects, these *velvet* family regulators may act globally at a higher hierarchical level, influencing multiple biological processes in many fungal species. The evidence that VosA is a transcription factor, combined with the observation that VeA, VelB, and VosA all localize in the nucleus at certain developmental stages (Ni and Yu, 2007; Stinnett et al., 2007; Bayram et al., 2008a), has led to the proposal that VeA family proteins define a new class of fungus-specific transcription factors. Additional study of *velvet* family proteins will further illuminate the molecular mechanisms connecting fungal morphogenesis and metabolism.

INDIRECT REGULATORS OF CONIDIATION: HETEROTRIMERIC G PROTEIN ELEMENTS

Heterotrimeric G proteins are conserved in all eukaryotes, and G protein signaling plays crucial roles in sensing and responding to various external signals such as nutrients, hormones, physical or chemical stimuli, and stresses from the external environment. The basic unit of G protein signaling includes a G-protein-coupled receptor, a heterotrimeric G protein consisting of three subunits, Gα, Gβ, and Gγ, and an intracellular effector that produces a second messenger (Lengeler et al., 2000; Neves et al., 2002; see also chapter 5, this volume). Upon binding of ligands,

G-protein-coupled receptors interact with inactive heterotrimers. This leads to the replacement of GDP bound to Gα for GTP and dissociation of Gα-GTP from the Gβγ heterodimer. Gα-GTP and Gβγ can act on downstream effector proteins to propagate the signals (Vaughan, 1998; McCudden et al., 2005; Yu, 2006).

RGSs negatively control the intensity and duration of the signal transduction by facilitating the conversion of active Gα-GTP into inactive Gα-GDP (Chidiac and Roy, 2003; McCudden et al., 2005). Phosducin-like proteins affect G protein signaling by acting as molecular chaperones during Gβγ assembly and by contributing to maintaining normal levels of Gβ and Gγ subunits (Kasahara et al., 2000; Knol et al., 2005; Lukov et al., 2005; Yu, 2006). In fungi, several pathways transmit and propagate the G-protein-mediated signals inside the cells (Morris and Malbon, 1999; Feldbrügge et al., 2004; McCudden et al., 2005; see also chapter 5).

G protein signaling plays important roles in cell growth, mating, cell-cell interaction, morphogenesis, chemotaxis, virulence, and secondary metabolite production in fungi (Bölker, 1998; Lengeler et al., 2000; Feldbrügge et al., 2004; Yu and Keller, 2005; Li et al. 2007). In aspergilli, most G protein signaling components indirectly affect conidiation.

G Proteins and RGS Mediate Developmental Regulation in *A. nidulans*

A bottleneck in understanding the upstream regulation of conidiation in *A. nidulans* was determining the function of *flbA* during conidiation. This was solved by identification and characterization of *fadA* (fluffy autolytic dominant), encoding a Gα subunit for a heterotrimeric G protein (Yu et al., 1996). *fadA* is a dominant activating mutation that results in a fluffy-autolytic phenotype almost identical to that of Δ*flbA*. A series of studies revealed that FadA-mediated signaling promotes hyphal growth and inhibits both asexual and sexual development and production of ST. Moreover, it was found that FadA signaling is in part transduced via cyclic AMP (cAMP)-dependent protein kinase A (PKA) (Yu et al., 1996; Hicks et al., 1997; Shimizu and Keller, 2001). FlbA is an RGS protein that plays a crucial role in controlling FadA-mediated vegetative growth signaling, likely by acting as a GTPase-activating protein for FadA (Yu et al., 1996). Both *flbA* loss-of-function and constitutively active FadA mutations that result in loss of intrinsic FadA GTPase activity result in a similar phenotype, i.e., uncontrolled accumulation of hyphal mass (Yu et al., 1996, 1999). Conversely, overexpression of *flbA* or the dominant interfering $FadA^{G203R}$ mutation leads to limited hyphal growth and hyperactive conidiation (Lee and Adams, 1994b; Yu et al., 1996). Later, a series of studies identified SfaD (Gβ) (Rosén et al., 1999), GpgA (Gγ) (Seo et al., 2005), and PhnA (a phosducin-like protein) (Seo and Yu, 2006). These G protein components function during vegetative growth signaling (see Yu et al., 2006). Moreover, these components are all indispensable for sexual-fruiting-body formation in *A. nidulans* (reviewed by Yu, 2006).

Importantly, while mutational inactivation of any one of these G protein components restores conidiation of the Δ*flbA* mutant to a certain level, no mutation can bypass the need for FluG during conidiation. These results corroborate the idea that the role of FlbA in asexual development is indirect and lead to the current model: vegetative growth and conidiation signaling are independent, and both inhibition of growth signaling and activation of development-specific functions must occur in order for development to proceed in *A. nidulans* (Fig. 9A). The second Gα-RGS pair (GanB-RgsA) has been shown to play a key role in regulating conidiation, conidial germination, and stress response (Han et al., 2004).

Conserved Roles of G Proteins and RGS in *A. fumigatus*

The corresponding *A. fumigatus* homologues of the abovementioned *A. nidulans* signaling elements show extremely high amino acid level identity (see Yu et al., 2006). To test whether conserved genetic inputs give rise to conserved cellular responses, Mah and Yu (2006) examined the functions of AfFlbA and GpaA (FadA homologue) in *A. fumigatus*. They generated the null Af*flbA* mutant and as well isolated 14 additional loss-of-function Af*flbA* mutants by employing a chemical mutagen. Phenotypic analysis revealed that loss of Af*flbA* function is associated with increased hyphal proliferation during the early phase of colony growth (up to 2 days), and reduced levels of conidiation and conidial pigmentation. In addition, unlike wild-type strains, $Af\mathit{flbA}^{-}$ mutants failed to produce conidiophores in liquid-submerged culture conditions. Finally, as observed for AfFluG, AfFlbA is necessary for proper expression of Af*brlA* and normal levels of conidiation. These observations led to the conclusion that AfFlbA inhibits hyphal proliferation, which in turn stimulates development in *A. fumigatus*. However, in contrast to *A. nidulans*, loss of Af*flbA* function does not block conidiation completely or lead to autolysis in *A. fumigatus*. This may be due to the presence of multiple mechanisms activating conidiation in *A. fumigatus*, which may alleviate the indirect need for AfFlbA during conidiation.

Is GpaA (the FadA homologue) the primary target of AfFlbA action? This question was answered by generating various mutant alleles including $gpaA^{Q204L}$ and $gpaA^{G203R}$. Mah and Yu (2006) found that, similar to the effects caused by ΔAf*flbA*, transformation of wild-type strains with the $gpaA^{Q204L}$ allele led to hyphal proliferation and reduced sporulation in a dominant manner without autolysis. Moreover, ectopic integration of the dominant interfering $gpaA^{G203R}$ allele restored conidiation in an $Af\mathit{flbA}^{-}$ mutant to wild-type levels, indicating that inactivation of GpaA signaling could bypass the role of AfFlbA in conidiation. Collectively, the study demonstrated that the primary roles of FadA/GpaA and FlbA/AfFlbA in controlling growth and development are conserved in aspergilli.

The Role of cAMP-PKA in Controlling Conidiation

The G protein-cAMP-dependent PKA signaling pathway plays a critical role in controlling fungal growth, development, nutrient sensing, mating, stress response, secondary metabolism, and pathogenicity. In *A. nidulans*, PkaA (primary) and PkaB (minor) represent the sole PKA catalytic subunits, and they play overlapping and opposite roles in diverse biological processes (Shimizu and Keller, 2001; Ni et al., 2005). FadA-dependent vegetative growth signaling is in part transduced via PkaA. The fact that GanB, SfaD::GpgA, and PkaA are required for proper germination of conidia (Chang et al., 2004; Lafon et al., 2005) indicates that PkaA is likely activated by both GanB and FadA. As PkaA is a key downstream element in the FadA-mediated growth signaling pathway, loss of *pkaA* function resulted in limited hyphal growth coupled with hyperactive conidiation and suppression of the fluffy-autolytic phenotype caused by Δ*flbA* (Shimizu and Keller, 2001). Furthermore,

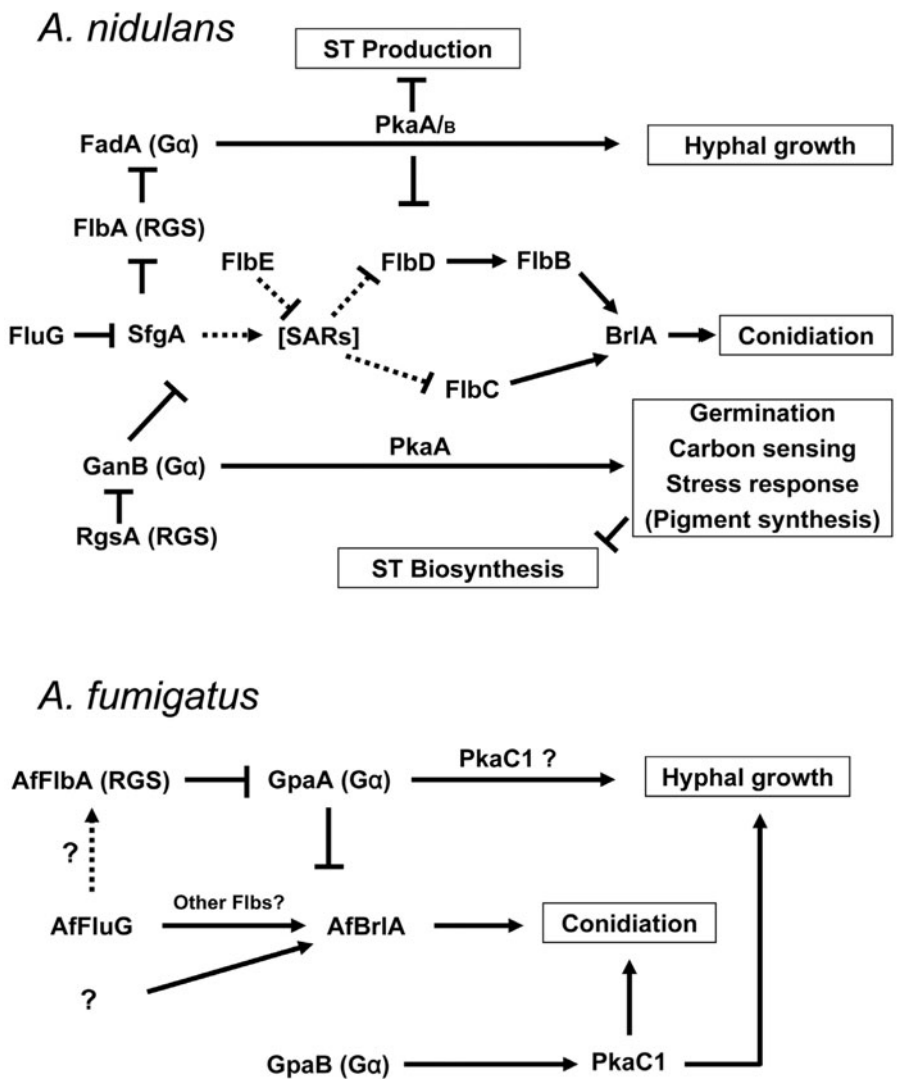


FIGURE 9 Models for growth and developmental control in *A. nidulans* and *A. fumigatus* (adapted and modified from Yu et al., 2006). Current models depicting upstream and downstream regulation of hyphal growth and conidiation in the two aspergilli are presented. Note that the roles of PkaA and PkaC1 in regulating conidiation are opposite.

overexpression of *pkaA* led to elevated hyphal proliferation and reduced sporulation. Collectively, a cAMP/PKA signaling cascade plays a major role in activating vegetative growth and repressing conidiation downstream of G proteins in *A. nidulans* (Fig. 9A).

The *A. fumigatus* primary PKA, PkaC1 (86% identity to PkaA) plays a major role in controlling growth and development (Liebmann et al., 2004). PkaC1 is necessary for proper vegetative growth, germination, and conidiation. Liebmann et al. proposed that GpaB, AcyA (adenylate cyclase), and PkaC1 constitute a major signaling cascade controlling vegetative growth, development, and virulence (Liebmann et al., 2003, 2004). Particularly, it was shown that the cAMP-PKA network is necessary for proper expression of *pksP*, encoding a polyketide synthase that is involved in the biosynthesis of the conidial pigment, 1,8-dihydroxynaphthalene-like pentaketide melanin, conferring resistance to phagocytic cell destruction in the host. The potential participation of PkaC1 in the GpaA signaling branch remains to be investigated. Taken together, despite high amino acid level identity, the role of a G protein and PKA in controlling conidiation can differ between aspergilli. A current model depicting regulation of vegetative growth and development in *A. nidulans* and *A. fumigatus* is presented in Fig. 9.

CONCLUSIONS AND PROSPECTS

Although asexual sporulation is the most common reproductive mode of many filamentous fungi, little is known about the regulatory mechanisms controlling this process. In this chapter, we have discussed our current understanding on the genetic regulation of *Aspergillus* conidiation, with the focus on the model fungus *A. nidulans*. A series of excellent studies have identified and characterized a number of genes (*fluG*, *flbB*~*flbE*, *brlA*, *abaA*, and *wetA*)

that activate conidiation in *A. nidulans*. Mutational inactivation of these developmental activators results in a lack or delay of conidiation in *Aspergillus* species. Accordingly, it has been proposed that conidiation occurs via the activities of multiple positive regulators. However, recent genetic studies have identified a key upstream negative regulator, SfgA, and revealed that a primary molecular event responding to FluG activity for the commencement of conidiation is to remove repression mediated by SfgA. The genetically programmed negative regulation of developmental transition may explain how fungal cells initially undergo vegetative growth for a certain period, while controlling precocious progression of conidiation. Moreover, identification of a multicopy repressor of conidiation revealed that the maturation of both asexual and sexual spores requires a novel gene (*vosA*). VosA plays two principal roles: (i) coupling sporogenesis and trehalose biogenesis to complete spore maturation and (ii) exerting negative-feedback regulation of developmental specific genes by repressing the expression of *brlA* encoding the key activator of conidiation in *Aspergillus*. VosA and related *velvet* proteins are found to be crucial global regulators for fungal development and metabolism. These new findings led to the current model that regulation of asexual development in *Aspergillus* occurs via balanced activities of multiple positive and negative regulators.

What lies ahead? The main challenge for comprehending the detailed molecular mechanisms that regulate conidiation is the identification of target genes controlled (or affected) by these positive and negative regulators. While genetic studies, e.g., recessive or dominant suppressor analyses, could be useful, these certainly have their limitations. Comparative genomics and functional genomics employing microarrays, ChIP-chip, protein two-dimensional gel electrophoresis, and the yeast two-hybrid system may be better suited to elucidate the regulatory cascades that are crucial for the regulation of vegetative growth, initiation, progression, and termination of conidiation, spore maturation, and toxin production in various aspergilli.

We express sincere appreciation to many fungal biologists who contributed to our current understanding of the regulation of conidiation in aspergilli. This work was supported by the USDA CSREES Hatch project (WIS04667) and National Science Foundation (IOS-0640067) grants to J.H.Y.

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The Conidium

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A conidium is an asexual spore formed from the apex or side of a conidiogenous cell that is not derived by cytoplasmic cleavage like sporangiospores or ascospores. The conidiogenous cell or a cluster of such cells with or without supporting hyphae is called the conidiophore. Conidia are nonmotile, in contrast to the uniflagellate spores produced by the Chytridiomycota by cytoplasmic cleavage (Fig. 1) (James et al., 2006). The sporangiospores produced in sporangia in the Zygomycetes are not conidia, although many species display remarkable structural complexity and prolific spore production (Fig. 2) (Jennessen et al., 2008). In discussing zygomycete sporulation, it is sometimes difficult to distinguish that the spores produced singly on sporangiola in some species are not conidia. However, it appears that these are single-spored sporangia surrounded by a thin sporangial wall and so are not true conidia (Poitras, 1955). Other zygomycete "conidial" forms may exist, but such a debate is beyond the scope of this chapter. *Coccidioides immitis* produces conidia during saprophytic growth. However, it also produces during infection of animals asexual endospores in sporangia-like spherules that do not fit the definition of conidia (Huppert et al., 1982).

Formation of conidia is common in the Ascomycetes and is also found in the Basidiomycetes, although a term specific to the system being examined is often substituted for the generic "conidia." For example, the conidia of *Coprinopsis cinerea* (*Coprinus cinereus*) are called oidia (Polak et al., 2001), whereas the multiple asexual spore types associated with different stages of the life cycles of the rust fungi have been given specific names that are more informative than the general term.

Saccardo (cited by Alexopoulos et al., 1996) used the morphologies of the conidiophore and conidia as taxonomic characters for the asexual ascomycetes (called the imperfect fungi or the Deuteromycetes). Memorization of the Saccardoan terms for the different conidia and conidiophore types, as well as forms of spore ontogeny, is still an important rite of passage in the training of mycologists. A good dictionary for these terms has been published (Ulloa and Hanlin, 2000), and illustrations of the major morphological features of conidiophores and conidia used in classification schemes can be found in descriptive publications (Hughes, 1953). The diversity of functions of conidia is reflected in their morphology. Although information is still limited, the data are beginning to reveal common and unique genes and proteins expressed during conidium formation in different conidial types. In many species, more than one type of conidium may be produced by an individual for different purposes. An intriguing question concerns the evolutionary origins for these developmental pathways. Are conidia and the regulatory pathways controlling their formation in extant species the result of divergence from an ancestral sporulation process? Or has conidiation evolved independently many times with little in common between sporulation processes in different fungi or even for different sporulation pathways within a single species?

FORM AND FUNCTION IN WATER, SOIL, AND AIR

Some fungi have adapted to life in water, and many shapes and sizes of single- and multiple-celled structures can be observed in these fungi. They are called aquatic hyphomycetes. The term hyphomycetes refers to fungi that do not produce conidia encased within fruiting bodies. Many of the aquatic hyphomycetes have no known sexual stage; however, molecular studies show that they are a phylogenetically diverse group, with most isolates belonging to the Ascomycetes and fewer to the Basidiomycetes. One group of aquatic fungi, often referred to as the Ingoldian fungi (Ingold, 1942), can produce branched conidia (Fig. 3) that allow the spores dispersed through water currents to attach to submerged vegetation (Webster, 1959). Another major morphological group, the helicosporous fungi, produce coiled conidia in two or three dimensions (Fig. 4). A number of these species trap air bubbles within their barrel-shaped conidia to promote buoyancy. Phylogenetic analysis demonstrated that the coiled conidial form arose independently in at least six different fungal lineages (Tsui and Berbee, 2006).

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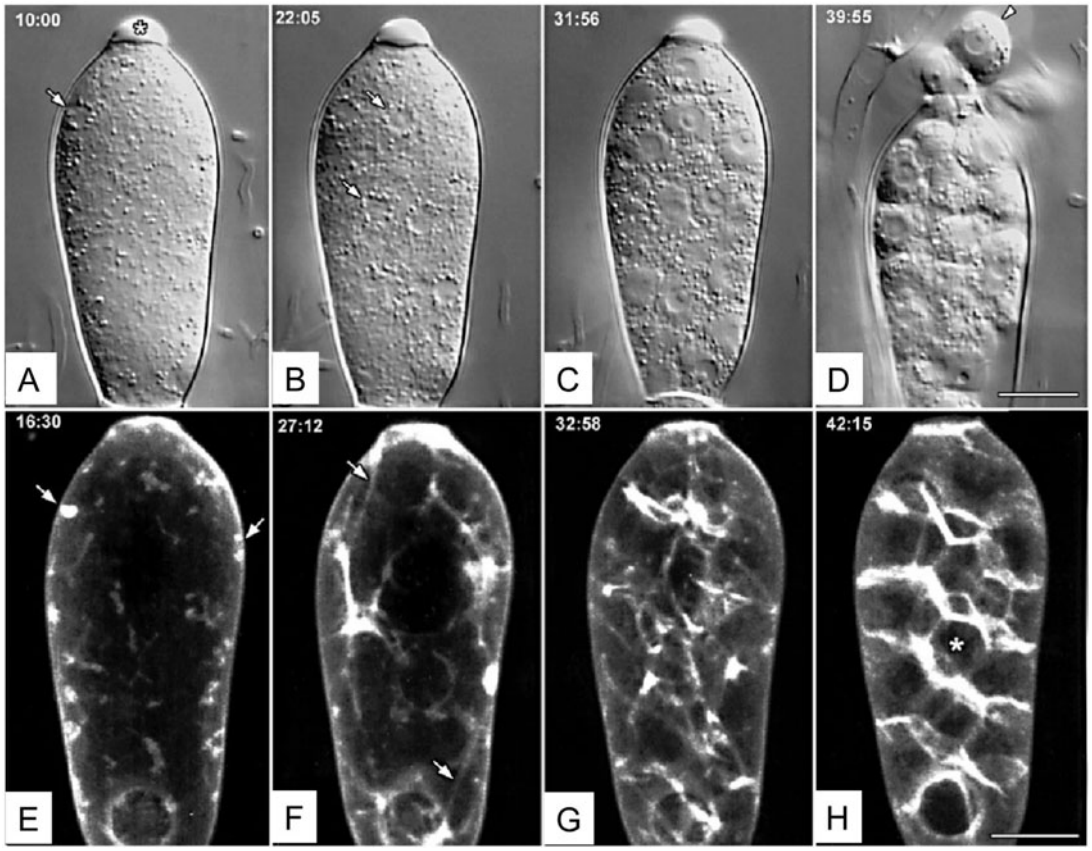

FIGURE 1 Cytoplasmic cleavage in zoosporogenesis. The cytoplasm of the sporangium is divided into membrane-bound compartments that give rise to uniflagellate zoospores. Control treatments of zoospore formation in living sporangia of *Allomyces macrogynus* were observed with video-enhanced differential interference contrast optics (A through D) and confocal microscopy after FM4–64 staining (E through H). Minutes and seconds in the upper left corners indicate postinduction times. Scale bars = 10 mm. (A through D) Nuclei were located in the cortical cytoplasm during early stages of zoospore formation (arrow, A) and by 20 to 30 min postinduction were positioned throughout the cytoplasm (arrows, B). Cytoplasmic domains became distinct (C), and eventually the papillum (asterisk, A) deliquesced and mature zoospores (arrowhead, D) maneuvered out of the sporangium into the surrounding medium. (E through H) By 12 to 20 min postinduction, areas of increased fluorescence (arrows, E) were observed along regions of the plasma membrane. Cleavage elements initially extended from these regions within the sporangial cortex (arrows, F) followed by a rapid elongation inward toward the center of the sporangium (G). By 40 to 50 min postinduction, zoospore initials were delimited into polyhedral cells (asterisk, H). Reprinted from Lowry et al. (2004) with permission from the Mycological Society of America.

Many fungal species reside in soil, often in association with plant roots. Different structures and cell types, including conidia, can serve as resting cells until environmental conditions are favorable for growth. It is reasonable to suggest that soil can serve as a reservoir for conidia that serve as propagules for infection of insects, mammals, and plants. However, the overall role played by conidia in the soil is not well understood.

It seems clear that conidia of *C. immitis* in the soil can serve as inocula for human hosts (Huppert et al., 1982). Similarly, one might expect that the insect pathogen *Metarhyzium anisopliae* rests in the soil as conidia associated with insect cadavers, until a new potential host is encountered. However, when tracked in the field, it appears that the fungus is most readily found associated with plant roots (St. Leger, 2008). This suggests that the fungus may have a close relationship with plants and may protect plants from insect damage. As another example of the types of interactions that may be common in the soil environment, conidia of the pea pathogen *Nectria haematococca* are induced to germinate in response to flavonoid compounds produced by its host. These same compounds also can induce nodulation gene expression in symbiotic bacteria, again illustrating the complex ecology in the soil (Straney et al., 2002).

In general, fungi dispersed in air, such as *Aspergillus*, *Neurospora*, and *Botrytis*, produce conidia that are rounded, small, pigmented, and hydrophobic. Dispersal may be a passive event subject to the winds or other disturbances that launch spores into the air. However, even in apparently passive release, such as for *Botrytis*, conidiophore movements induced by hygroscopy can provide enough energy to dislodge conidia (Meredith, 1973). Active release

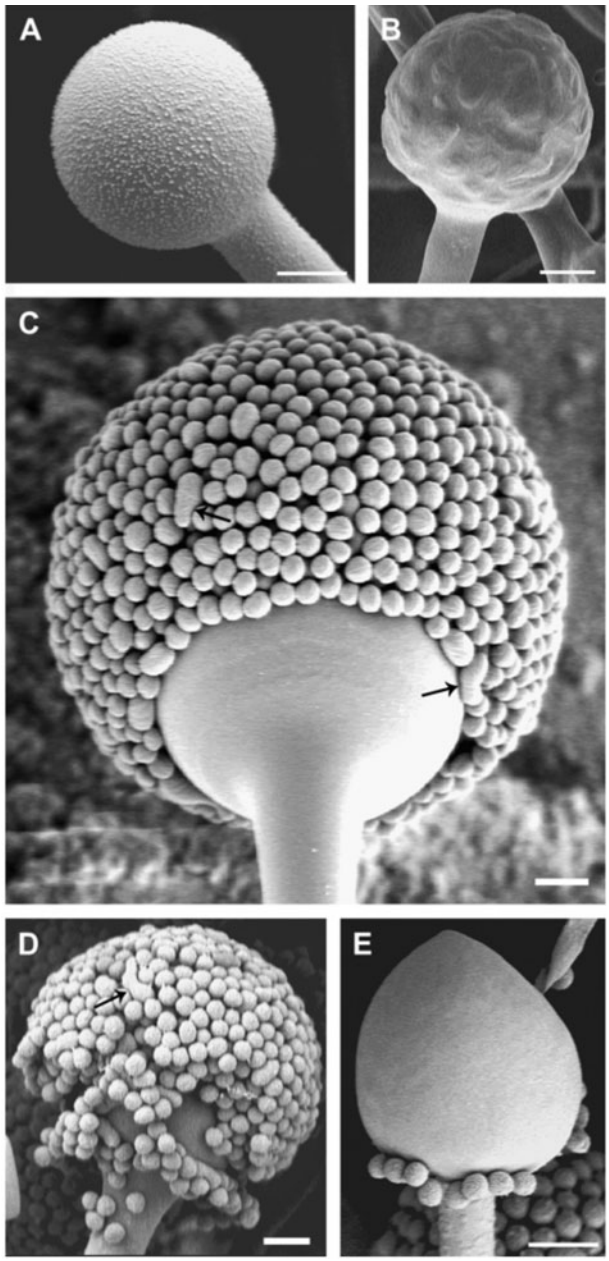

FIGURE 2 Developmental stages of *Rhizopus* sporangiophores and sporangia as seen under low-temperature scanning electron microscopy. (A) Immature sporangiophore; (B) young sporangiophore with developing spores in sporangia; (C) mature sporangium with sporangiospores; (D) sporangiospores starting to be released from an old sporangium; (E) columella with only a few spores left. Arrows indicate examples of anomalies in sporangiospore size and shape. (A) *R. rhizopodiformis* CBS 102277; 3 days, MEA; (B) *R. oligosporus* CBS 339.62; 3 days, malt extract agar (MEA); (C) *R. homothallicus* CBS 111232; 4 days, oatmeal agar; (D through E) *R. rhizopodiformis* CBS 536.80, 12 days, MEA. Bars, 10 μm. Reprinted from Jennessen et al. (2008) with permission from Elsevier.

of cells in fungi has been most carefully analyzed for sexual spore discharge (Yafetto et al., 2008). However, active spore launching has also been observed for conidia, either through rapid cell movements (Meredith, 1973) or, in the case of *Magnaporthe oryzae*, through the hypothesized bursting of the stalk cell attaching the conidium to the conidiophore (Ingold, 1964).

Raindrops can cause dispersal of dry conidia as the impact of the drop physically disturbs nearby conidiophores. Hydrophobic conidia may be carried on the surface of droplets. However, many spores are dispersed within water that arises from dew formation as well as rain. Conidia that are primarily splash dispersed often are formed in a mucilage (*Colletotrichum*, for example) that dissolves in water such that conidia are suspended in a water layer that can then be efficiently dispersed in microdroplets following the impact of a raindrop (Meredith, 1973; Fitt et al., 1989). *Colletotrichum* and other preferentially splash-dispersed species often produce their conidia within distinct fruiting bodies (Fitt et al., 1989). In many species, splash-dispersed conidia differ from wind-dispersed spores in having thinner cell walls, less pigmentation, elongated shapes, and hydrophilic surfaces. This is consistent with the view that they are protected from desiccation and other environmental hazards by their fruiting bodies or the mucilage that surrounds them until water becomes available for dispersal.

Animals and insects can also disturb aerial conidiophores or carry conidia to new locations. Insect vectoring of fungi is common. Bees can carry fungal conidia in their pollen baskets and have been shown to carry fully loaded baskets of *Neurospora* conidia (Shaw, 1998). The oak wilt pathogen, *Ceratocystis fagacearum*, produces a sweet odor from the spore mats it produces on infected trees. Beetles attracted to the spore mats pick up conidia and carry them to a new host (Appel et al., 1990). *Epichlöe* provides another example wherein conidia, acting as spermatia, are carried by an insect to another site to fertilize the opposite mating type (see chapter 41). Insect-to-insect transmission of entomopathogenic fungi also occurs.

MORPHOGENESIS

A description of the cellular and molecular events that occur during conidiophore and conidial formation is needed to completely define a developmental sequence. Ultimately, fungal cell growth and fungal cell shape determination, as well as formation of multicellular structures, involve the appropriately regulated growth required to deposit the proper cell wall material at the right place and time. A discussion of hyphal growth and polarity determination, cell cycle, signal transduction, etc. (see chapters 16 through 20), is integral to understanding the cell biology of development. However, studies of the basic cell biology of hyphal growth have generally not considered conidial development. Extension of these basic cell biology questions is needed to explain not only how the Spitzenkörper or polarisome is organized to produce hyphal growth or branch formation, but also how the distinct morphologies that make up conidiophores and conidia are produced. Some of the more interesting cell biology questions may relate to how conidiophore and conidial development differ from the basic polarized growth of hyphae.

A brief review of some of the morphological descriptions that have been used to imperfectly describe cell ontogeny illustrates the different growth modes involved in conidial development. Blastic development refers to cell growth that blows out from a narrow region with elongation and swelling (budding) before delimitation by a septum. If the outer cell wall of the spore is contiguous with the outer wall layer of the progenitor cell, it is termed holoblastic, as is the case for macroconidiation in *Neurospora crassa* (Springer and Yanofsky, 1989) (Fig. 5A and B). Enteroblastic conidia

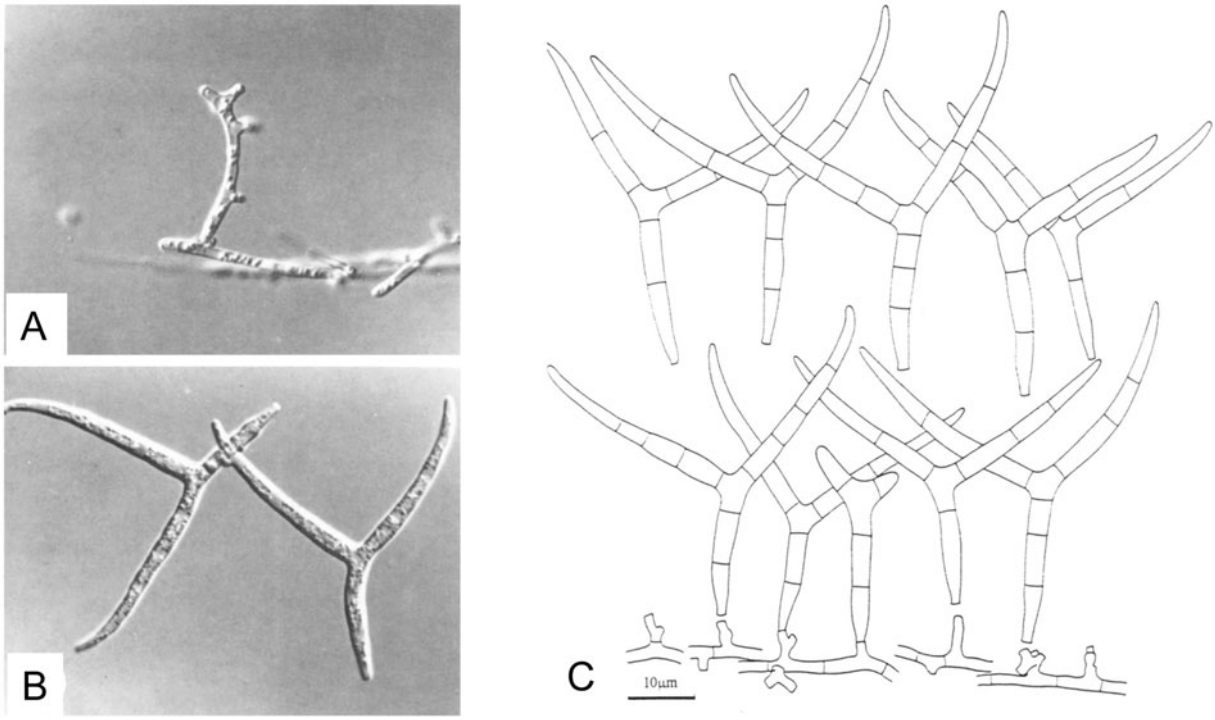

FIGURE 3 Conidial development in *Trinacrium subtile*, Reiss (1852). (A) The conidia are produced in a sympodial fashion, and the sites of conidial production are apparent after detachment from the conidiogenous cell. (B) Note the Y-shaped conidia of this Ingoldian aquatic hyphomycete. (C) Illustration of conidia and conidiogenous cells. Images provided by S. S. Tzean, National Taiwan University.

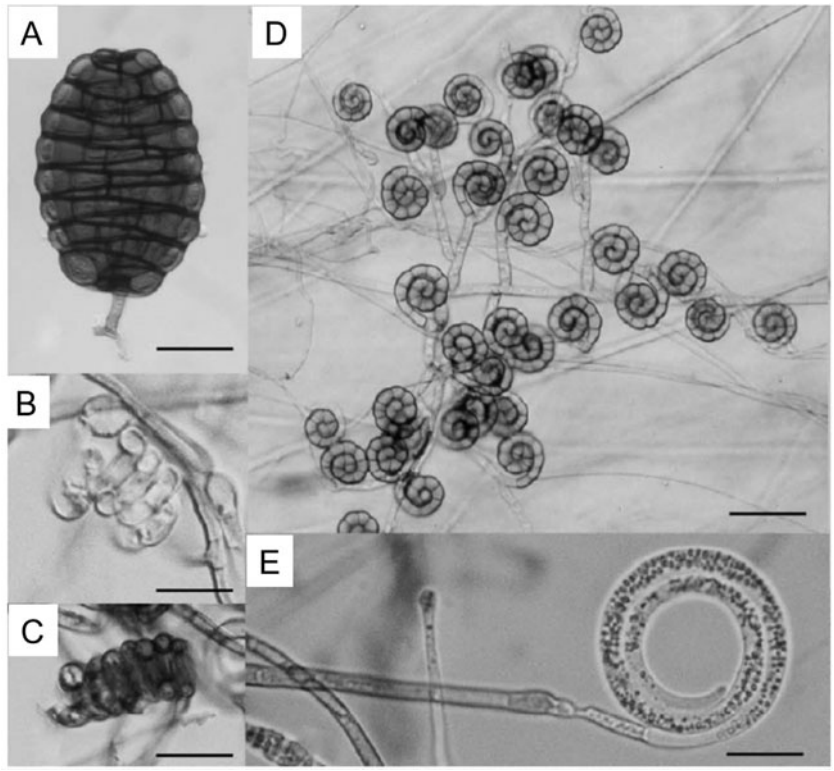

FIGURE 4 Coiled conidia of helicosporous fungi. (A and E) Conidia from the Tubeufiaceae; (B through D) conidia from other lineages. The conidia in panels A through C are three-dimensional and doliiform (barrel-shaped); those in panels D and E are two-dimensional and planate. Species represented are *Helicoon gigantisporum* (A), *Helicodendron tubulosum* (B and C), *Helicoma olivaceum* (D), and *Helicomyces roseus* (E), with a conidium developing from a conspicuous, erect conidiophore. Images are differential interference contrast micrographs; scale bar for panels A, B, and D, 30 μm; for panel C, 25 μm; for panel E, 15 μm. Reprinted from Tsui and Berbee (2006) with permission from Elsevier.

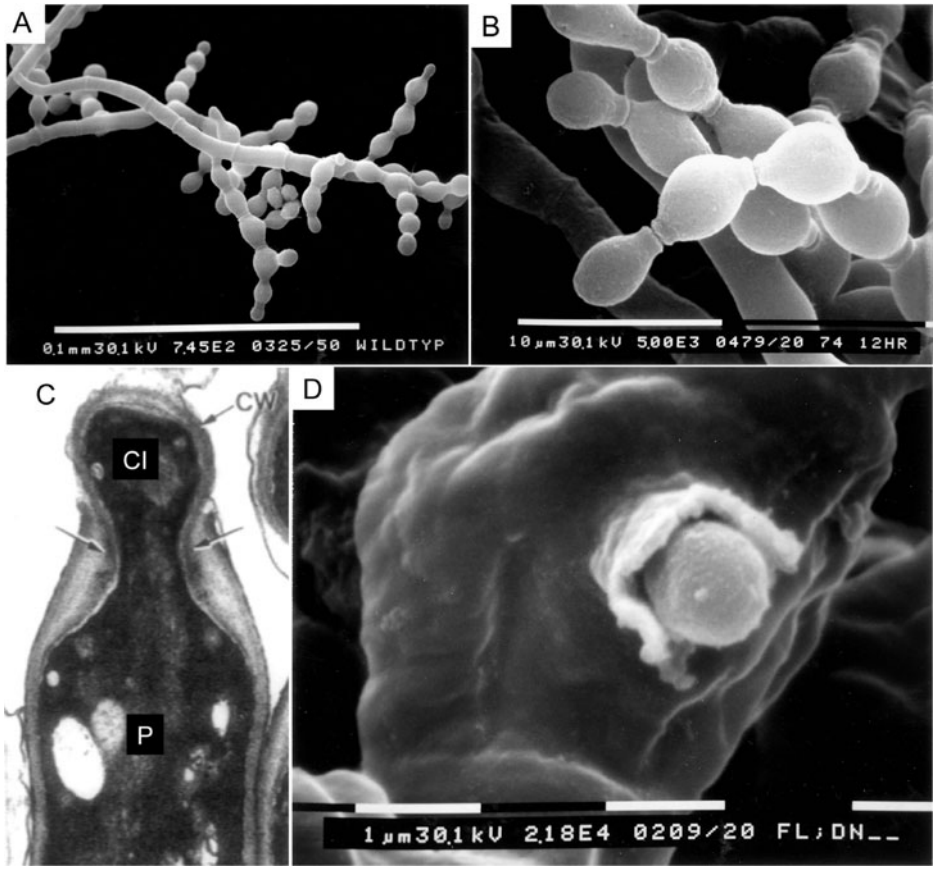

FIGURE 5 Ontogeny of blastic and thallic conidia. (A) Conidiophore of *N. crassa* showing holoblastic conidiation. Conidia are produced by repeated apical budding with an intact outer cell wall during macroconidium formation. (B) *N. crassa* holoblastic macroconidiation showing arthric separation of individual conidia. (C) Enteroblastic conidia development observed in the conidiophore of *A. nidulans*. The phialide, P, cell wall differentiates to form an inner wall layer (arrows) that forms a blastic conidium initial, CI, which pushes through the phialide cell wall to form the conidial cell wall (arrow labeled CW), distinct from the phialide cell wall layer. Photograph from Sewall et al. (1990) with permission from Elsevier. (D) Microconidia of *N. crassa* pushing through the phialide cell wall. Photographs for panels A, B, and D provided courtesy of Matthew Springer, University of California—San Francisco, and Oxford University Press (Davis, 2000).

arise from an inner wall layer that grows past the outer cell wall layer as it emerges from the conidiogenous cell (called a phialide), as is the case in *Aspergillus nidulans* (Sewall et al., 1990) (Fig. 5C). Repeated cell production basally pushes the older cell out, and chains of conidia may form. Blastic development differs from thallic development, in which a cell elongates without budding from a conidiogenous cell that may, or may not, subsequently swell after delimitation by a septum.

Arthric conidia are formed from a chain of cells delineated by septa that disarticulate into individual cells. Holoarthric spores are formed when the outer cell wall of the spore is derived from the cell wall produced during the elongation of the conidiogenous hypha. Enteroarthric conidiation refers to spores that form such that the outer cell wall of the spore is not contiguous with the cell wall of the conidiogenous hypha. The arthroconidia of *C. immitis* provide an example of this form of development. These terms can be combined in various descriptive combinations; for example, *N. crassa* macroconidia (Springer and Yanofsky, 1989) are formed in a blastic and arthric (arthroblastic) fashion (Fig. 5B). *N. crassa* also produces arthroconidia in which sections of the macroconidiophore have not undergone blastic growth (they are thallic) and break apart (arthric) into single-celled conidia (Springer and Yanofsky, 1989). This is distinguished from the microconidia of *N. crassa* (Maheshwari, 1999) that are produced by an enteroblastic process from a phialide very similar to conidial formation in *A. nidulans* (Fig. 5D).

The terms described above represent ideals, and the plasticity of development necessitates taking the view that variations on these themes may result in intermediate or mixed modes of development within species. This is problematic for classification if individuals constituting a species display variation such that they appear to differ in their modes of conidiation.

INDUCTION OF CONIDIATION

The environmental cues that induce conidiation have been selected to adapt to specific niches, but some common cues are a hyperoxidant state (e.g., exposure to air after growing

submerged in aqueous medium) and starvation. Exposure to air is a strong signal to induce conidiation for aerially dispersed conidia. The hyperoxidant state induced by exposure to air was shown to cause the oxidative inactivation of glutamine synthetase and NADP-dependent glutamate dehydrogenase in *N. crassa*, potentially connecting oxidative stress to nutrient signals (Toledo et al., 1994). Subsequently, many further studies have demonstrated the effect of modulating reactive oxygen species on induction of conidiation. Recent work has shown that NADPH oxidase function is needed for maximal levels of aerial hypha formation and conidium production. In addition, the enhancing effect on conidiation of mutation of a catalase gene of *N. crassa* depends on NADPH oxidase. Thus, both endogenous programmed regulation of NADPH oxidase activity and exogenous oxidative stresses may serve as signals for development (Cano-Dominguez et al., 2008).

Sensing of nutrients is important in induction of conidiation for many fungi, and transfer of cultures from nutrient sufficiency to carbon- or nitrogen-deficient media serves as a strong signal even in the apparent absence of oxidative stress. Additionally, direct responses to environmental signals such as light, temperature, and specific chemical cues from the environment serve to induce development. Fungi have mechanisms for sensing nutrient levels (chapter 30); however, it is unknown if nutrient-sensing pathways are manipulated as part of an endogenous developmental program. Light and temperature can be used to control the circadian clock, and in *N. crassa*, the induction of conidiation by the circadian rhythm is well documented (chapter 29).

One example of the light regulation of conidiation is provided by *M. oryzae* (Lee et al., 2006). In contrast to the case for *N. crassa*, where blue light stimulates conidiation, blue light inhibits aerial hypha formation in *M. oryzae*, and aerial development and conidiation are stimulated in darkness (Fig. 6A). Furthermore, the conidia are not released until exposure of the conidiophores to either blue or red (but not far-red) light (Fig. 6B). This detection of different

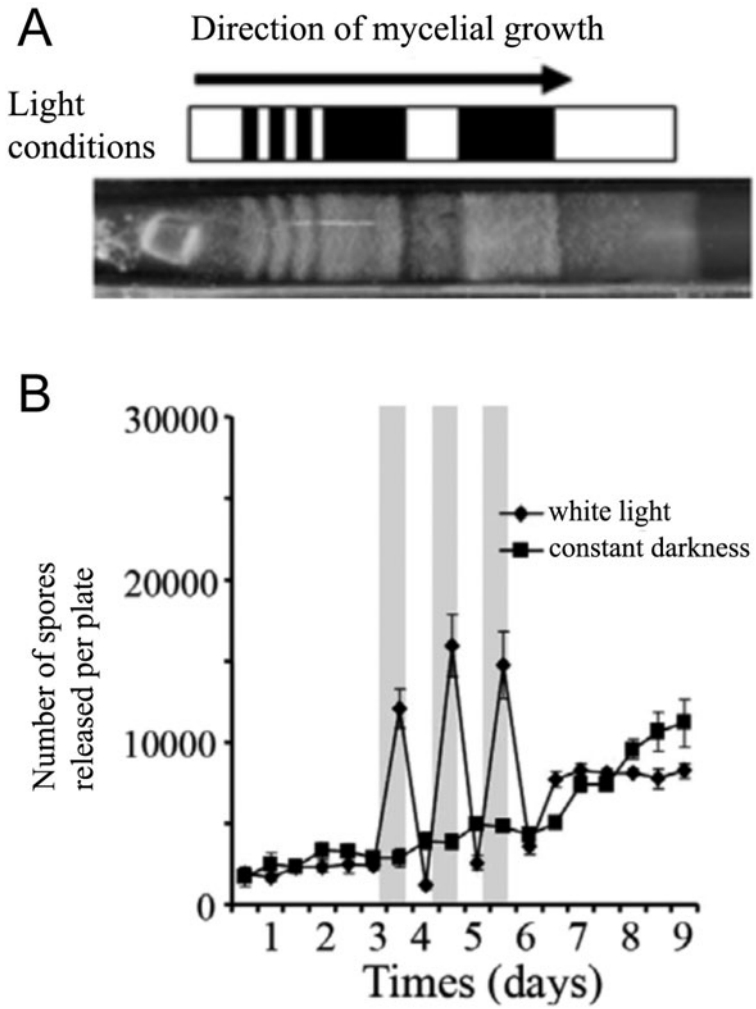

FIGURE 6 Effect of light on conidiophore development and spore release of *M. oryzae*. (A) Aerial development and conidium formation are repressed by light. *M. oryzae* growing (left to right) across a strip of medium and exposed to light (white boxes) and dark (black boxes). (B) Spore release activated by light. Cultures grown in constant darkness fail to release spores (line with boxes); 12-h exposure to light induces spore release (line with diamonds). Reprinted from Lee et al. (2006) with permission from Elsevier.

light wavelengths and the requirement for transitions from light to dark (and dark to light) for the behavior makes *M. oryzae* a particularly interesting example of light-responsive behavior in conidiation.

Another example of induction of a developmental program is provided by a phenomenon known as microcycle conidiation. This is the formation of conidiophores or conidia directly from another spore, and this phenomenon is observed in a number of fungal species (Hanlin, 1994). Microcycle conidiation occurs in response to nutrient conditions or a temperature treatment. Genetic variants that enter into microcycle conidiation more readily than the wild type have been noted (Maheshwari, 1991). In *N. crassa*, nutrient stress induces macroconidia to germinate directly to form macroconidiophores (Ton-That and Turian, 1978); in contrast, heat treatment causes microconidia and macroconidia to germinate directly to produce phialides and microconidia (Rossier et al., 1977). Thus, environmental conditions can serve as a switch to select which sporulation process is induced. The observation that developmental programs can be switched from one form to another during colony development is further strengthened by the fact that macroconidia form earlier in culture (2 to 3 days) than do microconidia (5 or more days) in *N. crassa* and once microconidium production begins, no new macroconidia are produced.

Despite the finding that some fungi, including some aspergilli, undergo microcycle conidiation, *A. nidulans* is well known for requiring a period of vegetative growth to achieve competence to permit conidiophore formation. Conidia must germinate and grow for approximately 15 to 20 h before the developmental program can be induced. The precise molecular signals are still being defined, and extracellular chemical signals appear to play an important role in this apparent cell-density-sensing mechanism. In *Aspergillus flavus*, the density of plating of conidia on culture medium determines the amount of conidia produced relative to sclerotia (densely packed melanized hyphal masses). Low-density inoculated cultures produce mainly sclerotia, while dense conidial inoculation maximizes production of conidia (Horowitz Brown et al., 2008). It was found that lipid signaling is involved in this developmental switch and this signaling is quite similar to the lipid-derived signaling that controls asexual versus sexual development in *A. nidulans* (Tsitsigiannis et al., 2004, 2005). Some of the genes required for development of competence have been identified, and aspects of developmental competence are discussed in chapter 35.

GENES EXPRESSED DURING CONIDIATION

Genes preferentially expressed during conidium formation have been identified by differential screening of cDNA libraries (Zimmermann et al., 1980; Berlin and Yanofsky, 1985). These studies identified a number of abundantly expressed genes. More recently, microarray studies (Kasuga et al., 2005; Breakspear and Momany, 2007) of the life cycle are beginning to permit global analysis of gene expression patterns. The general finding is that conidia accumulate a set of RNAs that are lost rapidly following germination. It is thought that these RNAs represent genes that encode proteins that accumulate in conidia or mRNA that is prepackaged for translation upon the breakage of dormancy. Early analysis of these microarray studies suggests that in some fungi the genes for housekeeping functions are represented at lower levels in dormant conidia than in growing cells.

Efforts to characterize the promoter regions of several of these genes have led to the discovery of greater complexity than might be expected for genes expressed specifically during conidiation. This may reflect that a number of the abundantly expressed genes may be important for stress survival, and they also may be expressed in response to a variety of stress conditions. Promoter analysis has been performed by preparing promoter fragments fused to reporter genes followed by integration into the genome at a specific site by homologous recombination. Comparison of several different promoter constructs is challenging because it has not been trivial to construct the appropriate strains for comparison. Despite this technical challenge, a number of studies have provided insight into general promoter structure, and some of these studies are highlighted below.

Hydrophobins are proteins that self-assemble into protein monolayers at hydrophobic and hydrophilic interfaces (Wosten, 2001). They possess eight cysteine residues that are arranged in a characteristic pattern. These proteins coat the surfaces of a number of aerial structures including conidia, and mutants lacking hydrophobins have a phenotype of being easily wettable in that water droplets soak the hyphae rather than being repelled, and the mutant hydrophilic conidia are easily suspended in water. Hydrophobin monolayers formed at air-water interfaces reduce the surface tension of water and may play a role in allowing the fungus to more easily grow into the air. In *A. nidulans* two hydrophobin genes, *rodA* (for "rodlet") and *dewA* (for "detergent wettable"), are expressed during conidiophore development and are regulated by BrlA (for "bristle"), a transcription factor that controls conidiophore morphogenesis and expression of many conidiation-specific genes. A binding site for BrlA in the *rodA* promoter has been identified (Chang and Timberlake, 1993). RodA protein contributes to hydrophobicity of the conidiophore and conidia, and *rodA* mRNA does not accumulate in dormant conidia to high levels. DewA is expressed late in development and is localized to the conidial wall but not in the cell walls of conidiophores or hyphae (Stringer and Timberlake, 1995). RNA for *dewA* accumulates in conidia, but *dewA* mRNA rapidly turns over during spore germination.

Macroconidia of *N. crassa* also are coated with a hydrophobin rodlet layer (Fig. 7). The regulation of the *eas* (for "easily wettable") hydrophobin gene has been studied intensively because of its complexity. Analyses of the regulation of *eas* by light, development, and the circadian clock have defined a number of *cis*-acting regulatory sites in the promoter region (Lauter et al., 1992; Bell-Pedersen et al., 1996, 2001; Rerngsamran et al., 2005). For example, regulation during conidiation depends on FL (for "FLUFFY"), a binuclear zinc finger transcription factor. A strong binding site for FL is located ~1,500 bp upstream of the EAS coding region, and this site plays a prominent role in developmental induction (Rerngsamran et al., 2005).

The *con-10* (for "conidiation") gene was one of the first conidiation-specific genes identified in *N. crassa* (Berlin and Yanofsky, 1985; Roberts et al., 1988; Roberts and Yanofsky, 1989) and has homologs in fungi, bacteria, and plants. The distribution of the gene is not uniform across members of the Ascomycetes, and the gene is absent in some lineages and is present in single or multiple copies in others. CON-10 is a small protein of 86 amino acids and is a homolog of the bacterial KGG stress response proteins (PFAM PF10685) that may protect bacteria from thermal and acid stress. In plants, the KGG domain occurs in some of the LEA (late embryogenesis abundant) proteins that accumulate in seeds.

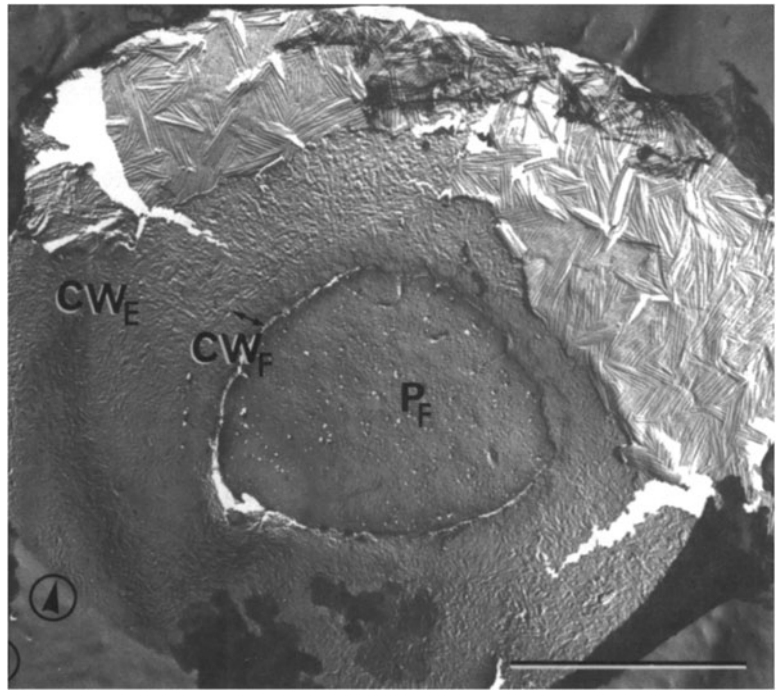

FIGURE 7 Deep etch of an *N. crassa* conidium showing hydrophobin rodlet layer. The initial fracture passed through the cell wall (CWF), exposing the plasma membrane face (PF). Etching then exposed the outside of the cell wall proper (CWE) and shows that in some regions the cell wall is covered by rodlets. Bar, 1 μm. Reproduced from Dempsey and Beever (1979) with permission from the American Society for Microbiology.

CON-10 protein and *con-10* mRNA are found in microconidia, macroconidia, and ascospores (Springer and Yanofsky, 1992; Lee and Ebbole, 1998a), and the gene is repressed in nondifferentiating hyphae (Yamashiro et al., 1996). In addition, *con-10* is repressed in the dark, induced within 1 min of exposure to the light (Corrochano et al., 1995), and regulated by the circadian clock (Lauter and Yanofsky, 1993). The gene is also regulation by carbon, nitrogen, salt stress, and heat shock (Watanabe et al., 2007), and these forms of regulation are more readily detected in the RCO-1 (for "regulator of conidiation") mycelial repressor mutant background (Lee and Ebbole, 1998b). One form of general repression of *con-10* is through RCO-1, and relief of this repression may allow for developmental activation. The sequence of RCO-1 (Yamashiro et al., 1996) reveals it to be a homolog of the Tup1p repressor of *Saccharomyces cerevisiae*. In *S. cerevisiae* Tup1p can alter chromatin structure as a mechanism to regulate gene expression (Malave and Dent, 2006). The *rco-1* ortholog in *A. nidulans*, *rcoA*, has been shown to affect chromatin structure at some promoters (Garcia et al., 2008).

The *cis*-acting elements responsible for some of these forms of regulation have been localized to specific regions of the promoter. In addition to regulation by environmental or developmental signals, there are at least two pairs of *cis*-acting elements that are important for generally enhancing the strength of the promoter (although there is apparent overlap with some light regulation elements). The two CGEs (for "*con-10* general enhancers") are 17-nucleotide sequences that bind to a protein found in extracts from conidiating cultures. No CGE binding protein was detected in mycelial extracts. Either of these two elements is sufficient for activation of the promoter, but deletion of both elements greatly reduces expression under all conditions of growth. One element is located 377 nucleotides upstream of the transcription initiation site, and the second CGE is located within the first intron of the gene (Corrochano et al., 1995; Lee and Ebbole, 1998a). The *con-10* promoter is also activated through two CRS-B (for "conidiation regulatory sequence B") elements (White and Yanofsky, 1993), both of which are needed for full activation during macroconidiation; however, the CRS-B elements do not appear to be required for activation of *con-10* expression in microconidiation (Lee and Ebbole, 1998a).

CON-6 is another small protein found in fungi (PFAM PF10346) that accumulates in *N. crassa* conidia along with its mRNA. *con-6* mRNA is first observed during macroconidiation about 6 to 8 h after developmental induction. This precedes induction of *con-10* expression by about 2 h. The *con-6* gene contains one CRS-B element. A protein that binds to the CRS-B element during vegetative growth but not during development was identified (White and Yanofsky, 1993), suggesting it serves as a repressor protein during vegetative development and plays a role in mycelial repression, much like RCO-1.

The *SpoC1* gene cluster of *A. nidulans* was discovered through analysis of conidiation-induced genes (Timberlake and Barnard, 1981; Gwynne et al., 1984; Miller et al., 1987; Aramayo et al., 1989). This cluster spans approximately 38 kb and contains an estimated 19 conidiation-specific genes expressed late in development, and their mRNA accumulates in conidia. The functions of the genes

are unknown, and deletion of the entire gene cluster does not result in a notable phenotype. It was found that when one of the genes was cloned and transformed ectopically into the genome, the basal level of expression was increased to a detectable level. The gene was still induced during conidiation at the ectopic site, suggesting that developmental induction was intact, but mycelial repression was defective. To test if the *SpoC1* cluster might represent a chromosomal region that is silenced during vegetative growth, the arginine biosynthetic gene *argB* was inserted into the *SpoC1* locus at three different sites by homologous recombination. The transformed gene was found to have low levels of expression in vegetative hyphae but restored expression levels during conidiation, supporting the view that *SpoC1* and possibly other developmentally regulated genes may be regulated by alterations in chromatin structure. It remains to be determined if gene clusters for conidiation-specific genes will be commonly found in *A. nidulans* or other fungi. Transcript profiles using *N. crassa* microarrays did not reveal large clusters of developmentally regulated genes during macroconidiation (Kasuga et al., 2005).

The promoters of important regulators of conidiophore development, such as *stuA* and *brlA*, have also been examined (Wu and Miller, 1997; Han and Adams, 2001). As with the examples shown above, the structures of these promoters are complex and made more complicated by the fact that both of the genes have two transcripts produced by overlapping promoters with alternative splicing. In the case of the *stuA* promoter, the region between the upstream and downstream transcription start sites contains BrlA binding sites that likely confer strong developmental regulation to the downstream promoter.

SIGNAL TRANSDUCTION IN CONIDIATION

A deficiency in adenylate cyclase activity in the *cr-1* mutant of *N. crassa* leads to derepression of conidiation at the expense of vegetative hyphal growth and the formation of nonconidiophore aerial hyphae. Likewise, mutation of a catalytic subunit of the cyclic AMP (cAMP)-dependent protein kinase PKAC-1 resulted in a phenotype very similar to the adenylate cyclase, with the mutant displaying reduced growth and constitutive conidiation (Banno et al., 2005). Heterotrimeric G proteins (and their receptors) are expected to play roles in signaling induction of conidiation through sensing environmental parameters such as carbon status, and GPR-4 coupled to GNA-1 appears to play this role for *N. crassa* (Li and Borkovich, 2006). Heterotrimeric G proteins positively regulate adenylate cyclase in *N. crassa*, and mutation of *gna-3* has the most dramatic effect of the three G-alpha subunit mutations in derepressing conidiation, suggesting that GNA-3 plays the greatest role in stimulating cAMP levels under vegetative growth conditions (Kays et al., 2000). Thus, signals that downregulate cAMP levels likely stimulate conidiophore morphogenesis. The Ras2 G protein is able to stimulate adenylate cyclase and mitogen-activated protein kinase (MAPK) pathways in *S. cerevisiae* to induce invasive growth (Mosch et al., 1999). If Ras plays a similar role in stimulating adenylate cyclase, one would expect mutations in Ras to activate conidiation. However, in *N. crassa*, a dominant negative allele for *bd* (for "band")*/ras-1* (Belden et al., 2007) and a null mutation of *ras-2* (Kanauchi et al., 1997) displayed reduced growth and a strong reduction in conidiation. The integration of Ras protein function into a model for control of the cAMP pathway and conidiation is incomplete.

The *N. crassa* MAPK MAK-2 is an ortholog of *S. cerevisiae* Fus3p and is required for female fertility. However, mutants also display a phenotype similar to adenylate cyclase or cAMP-dependent protein kinase mutants in their reduced vegetative growth and activation of conidiation (Pandey et al., 2004; Li et al., 2005). Similarly, mutation of the *N. crassa* homolog of *S. cerevisiae ste12* has a similar growth and conidiation phenotype. The cAMP pathway does not operate downstream of this MAPK, since added cAMP does not restore normal growth and development as it does with the adenylate cyclase mutant. It has not been determined if these are parallel pathways or if the cAMP pathway acts upstream of the MAK-2 pathway.

As discussed in chapter 35, mutation of *pkaA*, encoding the cAMP-dependent protein kinase of *A. nidulans*, leads to hyperconidiation. Thus, *A. nidulans* and *N. crassa* share a common repressive role for cAMP signaling in conidiation. Furthermore, a dominant activated allele of the G-alpha subunit, *FadA*, stimulates the protein kinase A pathway and reduces conidiation. The *flbA* (for "fluffy, low bristle A expression") gene encodes a regulator of G-protein signaling that downregulates FadA activity. Null mutants of *flbA* have elevated cAMP pathway activity due to deregulated FadA and therefore fail to produce conidia.

For *A. nidulans* and *N. crassa*, cAMP signaling clearly inhibits induction of conidiation. Conidiation in *M. oryzae* appears to be regulated in an opposite fashion by the cAMP pathway because mutation of the ortholog of *A. nidulans flbA* hyperactivates conidiation (Liu et al., 2007). Similarly, a dominant activated G-alpha subunit stimulates conidiation. Thus, although G-protein and cAMP signal transduction is used as part of the overall pathway controlling conidiation in all three fungi, the wiring of the circuit differs among them, just as the effect of light on conidiation can differ (stimulatory in *A. nidulans* and *N. crassa* and inhibitory in *M. grisea*).

CONSERVATION AND NOVELTY IN THE CENTRAL REGULATION OF CONIDIOPHORE DEVELOPMENT

The central regulatory pathway controlling conidiation in *A. nidulans* involves BrlA, AbaA (for "abacus"), and WetA (for "wet-white") to regulate production of vesicles, sterigmata (metulae and phialide cells), and conidia (Fig. 8). StuA (for "stunted") controls the height of the conidiophore. Usually the stalk cell is 100 μm tall, but in *stuA* mutants they are 20 to 50 μm tall. *stuA* mutant conidiophores have reduced complexity and produce conidia directly from the vesicle or stunted sterigmata. MedA (for "medusa") controls cell type determination. Mutants appear to reiterate cell types rather than terminally differentiate conidiophores, and they can even develop a new conidiophore that grows from the vesicle of a developing conidiophore.

Expression of BrlA controls conidiophore differentiation, and several genes, including the transcription factor-encoding genes *flbC* and *flbD*, appear to contribute to *brlA* expression. In addition, it is clear that *A. nidulans* has mechanisms for repressing conidiation under noninducing conditions (chapter 35). Although this is a simplistic view of the developmental events and the genetic regulation of

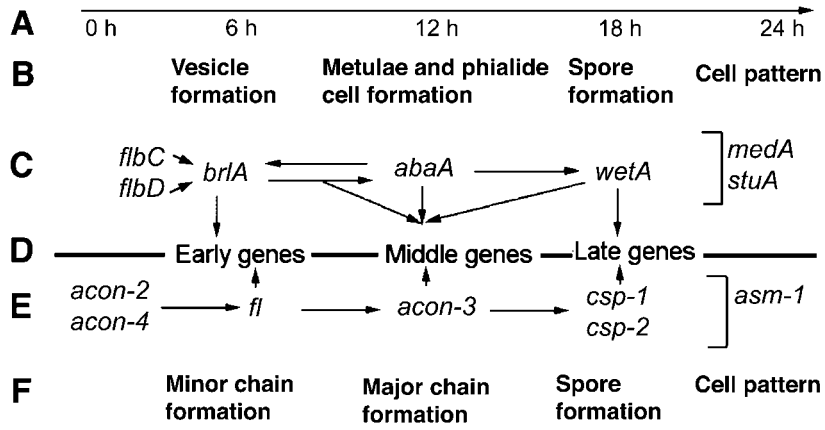

FIGURE 8 Analogous features in conidiation of *A. nidulans* and *N. crassa*. (A) Time coursean *A. nidulans* conidiation. (C) Regulatory genes controlling development in *A. nidulans*. (D) Timing of expression of conidiation-specific genes. (E) Regulatory genes controlling development in *N. crassa*. (F) Morphological landmarks in *N. crassa* conidiation. The *flb*C, *flbD*, *acon-2*, and *acon-4* play roles in activating the nonhomologous key regulatory genes *brlA* and *fl*. The role of *aba*A in *N. crassa* appears to be played by *acon-3*, which is required for major constriction chain formation. The *wetA*, *csp-1*, and *csp-2* genes are required for late stages of conidial morphogenesis and maturation. The *medA* and *stuA* (*asm-1*) genes play roles in conidiation across fungal species in cell patterning and conidiophore structure.aa

conidiation, it serves as a starting point for comparing analogous developmental stages between different conidiation pathways.

In *N. crassa*, FL plays the role analogous to BrlA in transitioning from aerial hyphae to formation of branched chains of blastic proconidia that mature by thallic disarticulation of the conidiophore (Bailey and Ebbole, 1998). Overexpression of *fl* induces conidiation in the wild type, as expected for a central regulator of the pathway. The *acon-2* and *acon-4* mutants are blocked in conidiophore development and may act before *fl*, since *fl* expression is blocked in *acon-2* and *acon-4* mutants (analogous to *A. nidulans flbC* and *flbD* [Fig. 8]). In addition, conidiation caused by overexpression of FL is blocked in an *acon-2* mutant background, suggesting that the role of *acon-2* is not simply to activate *fl* transcription. However, the *acon-2* mutant block to *fl* overexpression-induced conidiation is bypassed if the culture is grown under nitrogen starvation conditions. Defining the role of *acon-2* will require further characterization of the gene, which has yet to be cloned. The *acon-3* gene lies parallel to, or downstream of, *fl*. This model is based on the fact that *fl* expression is not blocked in the *acon-3* mutant (Bailey-Shrode and Ebbole, 2004). The recent cloning of *acon-3* and *acon-4* (unpublished) will help to determine how these genes act in the pathway. Two conidial separation (*csp*) mutant genes are known, and one of these genes, *csp-1*, was recently cloned with the aid of single-nucleotide polymorphism mapping and was shown to encode a transcription factor (Lambreghts et al., 2009). The *asm-1* gene is the ortholog of *A. nidulans stuA*, and conidiophores emerge from substrate hyphae and fail to produce tall aerial hyphae that support conidiophore development (Aramayo et al., 1996). Thus, it appears that the function of the *asm-1* gene is analogous to that of *stuA* of *A. nidulans*. Other, as yet uncloned, modifier genes that affect conidial size or branching pattern have been described (Springer and Yanofsky, 1989).

In *M. oryzae*, several mutant genes that alter conidial morphology and conidiation patterns have been found (Shi et al., 1998). One of these, *con7*, affects conidiation but also plays a critical role in infection-related development (Odenbach et al., 2007). Other genes that cause a strong reduction in conidiation and pathogenesis include a siderophore-deficient mutant gene (Hof et al., 2009) and MoSnf1p (Yi et al., 2008), the ortholog of *S. cerevisiae* Snf1p. Mutations affecting spore morphology, *smo1* (Hamer et al., 1989), and the developmental pattern of the conidiophore (*acr1*) have been identified (Lau and Hamer, 1998). Remarkably, the *acr1* (for "acropetal") gene is the ortholog of *A. nidulans medA*, and the mutant does not follow the normal pattern of terminally differentiating a conidium followed by further growth of the conidiogenous hypha before production of the next conidium. Instead, the tip of the first conidium serves as the site for budding of the next conidium from its apex (Fig. 9). This reiteration of cell types is analogous to the *medA* phenotype in *A. nidulans* and converts the conidiophore of *M. oryzae* to a new morphological class. This is a striking example of the morphological plasticity of fungi.

The orthologs of *medA* and *stuA* have also been characterized from *Fusarium oxysporum* (Ohara et al., 2004; Ohara and Tsuge, 2004). Mutation of the *medA* ortholog, *REN1*, fails to produce macroconidia or microconidia, and instead reiterates formation of a novel cell type (Fig. 9). The *FoSTUA* mutant has a stunted phenotype and does not produce an aerial conidiophore but can produce conidia directly from the substrate hyphae (Fig. 9). This presents a pattern of apparent conservation of gene use in the evolution of conidiation, and it appears that *medA* and *stuA* orthologs have very similar or analogous functions that have been conserved through divergence from a com-

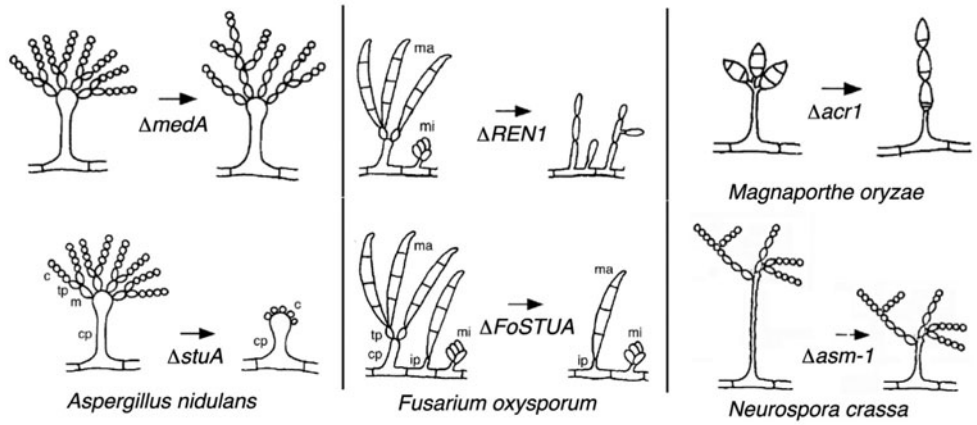

FIGURE 9 Conservation of developmental regulator function in evolution. The different fungal species display analogous effects on morphogenesis. The top row illustrates *medA*. In *A. nidulans*, *medA* mutants reiterate sterigmata and sometimes form new conidiophores from the vesicle. In *F. oxysporum*, the *ren1* mutants are unable to make either micro- or macroconidia and instead produce a reiteration of a new cell type. In *M. oryzae*, *acr* mutants fail to produce a sympodial arrangement of conidia and instead reiterate conidia, one on top of the preceding cell. This is called an acropetal mode of spore ontogeny. The bottom row illustrates phenotypic similarity in the *stuA* ortholog mutants across the fungi. In *A. nidulans*, the stalk cell is stunted and the sterigma is reduced (stunted). In *F. oxysporum*, the conidiophore is reduced so that conidiophores are not produced and macroconidia form directly from the substrate hyphae (intercalary phialides). Microconidium formation is not affected. In *N. crassa*, aerial hyphae are stunted, but conidiation is otherwise normal. Figure modified from Ohara et al., 2004, and Ohara and Tsuge, 2004.

mon ancestor (Fig. 10A). Both *stuA* and *medA* orthologous genes are found in all filamentous ascomycete fungi sequenced thus far. In addition, they are both required for sexual development in all species tested to date, further suggesting a conserved and perhaps ancient function for these genes.

BrlA plays a central role in regulating conidiation in *A. nidulans*, so an obvious question is to determine whether BrlA orthologs exist in other fungi and if these orthologs are involved in regulating conidiation. The answer is that BrlA is present in the aspergilli and closely allied lineages, such as *Penicillium* (Todd et al., 2003); however, more distantly related lineages do not possess a clear ortholog. Similarly, FL is a major regulator of macroconidiation in *N. crassa* and a likely ortholog is present in the closely related fungi *Podospora anserina* and *Sordaria macrospora* (which do not produce macroconidia). However, orthologs of FL are not obvious in more distantly related genera. This presents a second model for the evolution of regulatory genes for conidiation where novel regulators arise within lineages. Presumably, this occurs by duplication followed by divergence of the duplicated gene that then evolves as a developmental regulator found in closely related lineages (Fig. 10B). However, loss of a conidiation regulator, such as an ancient *brlA* gene from a common ancestor, cannot be formally excluded.

The *flbD* mutant of *A. nidulans* displays a long delay in conidiation because of lower *brlA* expression (Wieser and Adams, 1995). A clear *flbD* ortholog is present across the filamentous ascomycete fungal lineages, and the *N. crassa* ortholog *rca-1* (for "regulator of conidiation in *Aspergillus*") complements the conidiation defect of the *A. nidulans* mutant (Shen et al., 1998). However, mutation of the transcription factor encoded by *rca-1* does not affect conidiation in *N. crassa* but does have a minor effect on hyphal growth behavior. This defines a third model for the evolution of genes controlling conidiation, whereby an ancient gene encoding a transcription factor has been conserved, presumably for some conserved function, but *A. nidulans* has also co-opted the gene for use in controlling development (Fig. 10C).

Based on these different patterns of regulatory gene evolution, it is tempting to suggest that the conserved regulators of sporulation (*medA* and *stuA*) would likely be the ones that regulate the genes found to be expressed in common across fungi (hydrophobins, *con-6*, and *con-10*). The unique regulators (*brlA* and *fl*) might then have been recruited to regulate *medA* and *stuA* to carry out common functions. However, as discussed above, it is FL and BrlA that directly bind to the promoters of the hydrophobin genes expressed during conidiation. Thus, *brlA* and *fl* each independently evolved (and/or the promoters of *rodA* and *eas* evolved) to allow conidiation-specific regulation of these hydrophobin genes by the transcription factors so the hydrophobins could coat the surface of the conidium.

These models for the patterns of evolution of genes involved in regulating conidiation in different fungal lineages should also apply to the evolution of regulatory pathways governing independent conidiation pathways within a single species. For example, the *acon-2*, *fl*, *acon-3*, and *csp-1* genes discussed above, which control macroconidiation in *N. crassa*, do not affect microconidiation. However, there are conserved genes that have been selected to regulate conidiation, and further studies of these genes will provide a better understanding of what roles they play in either gene expression or controlling growth polarity. Principles gained from defining the evolution of

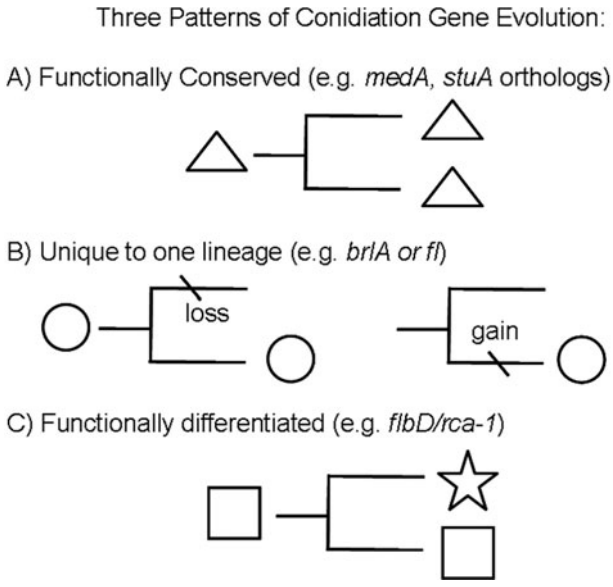

FIGURE 10 Patterns of gene evolution for regulators of conidiation. (A) Genes that are conserved across fungi and play a recognizably analogous role in conidiation. This pattern is represented by *medA* and *stuA* orthologs across a broad group of fungi. These genes also appear to be involved in sexual development in all fungi examined to date. (B) Lineage-specific regulators are genes that are unique to a particular fungal clade either through loss in most other fungal groups or gain in a particular clade. BrlA and FL exemplify this group. (C) Genes that are conserved across fungi may retain a common function, but in some lineages the gene has been adapted to also regulate a conidiation pathway. Alternatively, the ancestral gene was involved in development and this role was lost in some lineages. FlbD is an example of a protein that has retained its biochemical function across a broad evolutionary distance but plays different roles in the different fungal lineages.

conidiation pathways are likely to be informative for understanding the origins of other novel developmental pathways, such as pathogenesis.

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Magnaporthe oryzae and Rice Blast Disease

CHANG HYUN KHANG AND BARBARA VALENT

THE IMPACT OF BLAST DISEASE: PAST, PRESENT, AND FUTURE

Rice blast, caused by the haploid, ascomycetous fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) (Couch and Kohn, 2002), remains the most explosive and potentially damaging disease of the world's rice crop (Wang and Valent, 2009). Estimates are that the crops lost to the blast fungus would feed 60 million people annually, and strategies for durable, sustainable disease control remain elusive. The fungus infects all above-ground parts of the plant, with leaf blast (Fig. 1), neck rot, and panicle blast being responsible for significant losses in the field. In addition, wheat blast, caused by related *M. oryzae* strains, was identified in 1985 in Paraná State of Brazil (Igarashi et al., 1986), and it rapidly spread to the important wheat-producing regions of Brazil and Bolivia (Prabhu et al., 1992). Since then, blast has become a major wheat disease in this region (Urashima et al., 2004). The wheat blast fungus infects mainly wheat heads in the field, and fungicides that control rice blast are not effective in controlling wheat blast. Although wheat blast has not yet spread from South America, close relatives to the wheat isolates emerged in the early to mid-1990s to cause gray leaf spot (GLS), a severe turf grass disease, infecting perennial ryegrass and tall fescue in the United States and in Japan (Viji et al., 2001; Farman, 2002a; Tosa et al., 2004; Tredway et al., 2005). *M. oryzae* represents a growing agricultural threat.

The blast fungus executes diverse developmental processes, including the best-studied development associated with its asexual disease cycle (Fig. 2A through C). At least in laboratory studies, the fungus can also infect roots, executing a different developmental process characteristic of root pathogens such as the closely related wheat take-all fungus, *Gaeumannomyces graminis* (Sesma and Osbourn, 2004). The fungus produces hyaline to pale gray asexual conidia, each with three cells containing a single nucleus. Since the three nuclei in a conidium are identical, the fungus is routinely purified by isolation of a single conidium. The fungus is a member of the Pyrenomycetes, producing ascospores in unordered asci within perithecia (Fig. 2D). Fully fertile strains are self-sterile hermaphrodites, with compatibility for mating governed by alternate alleles of the mating-type locus *MAT1*. Small, crescent-shaped microconidia, 6 μm in length and 0.7 μm in width, are produced from phialides by some sexually fertile isolates (Kato et al., 1994). These microconidia have not been observed to germinate, and their role in nature is unknown.

For rice blast, differences in rice cultivar specificity were first distinguished by Sasaki in Japan in 1922, and he reported the first genetic analysis of resistance in rice (Ou, 1985). Since that time hundreds of races (or pathotypes) have been defined by their virulence spectrums on differential rice cultivars (Wang and Valent, 2009). Major effort has been directed toward identifying dominant resistance (*R*) genes in rice or wild relatives and toward developing blast-resistant cultivars. This has resulted in identification of >80 *R* genes that prevent disease by some pathogen races, as well as 350 quantitative trait loci (Ballini et al., 2008). Despite this wealth of *R* genes, genetic control has not been effective, due to the variability of the fungus and its ability to "defeat" introduced *R* genes within 1 to 2 years of their deployment in the field. Expectations are that blast disease will become even more serious under the conditions of agricultural intensification required to feed the growing world population (Wang and Valent, 2009). For example, both increased fertilizer use and decreasing water supply favor development of rice blast disease. Food security in the coming century depends on eliminating the potential of rice blast disease to cause catastrophic losses to rice production.

M. GRISEA SPECIES COMPLEX

The *Magnaporthe grisea* species complex includes pathogens of more than 50 grass species (Talbot, 2003). *Magnaporthe oryzae* was recently segregated as a distinct species from *M. grisea* based on a multilocus phylogenetic analysis and on mating properties of the strains (Couch and Kohn, 2002). *M. grisea* isolates are pathogenic on crabgrass, *Digitaria sanguinalis*, and related grasses, and *M. oryzae* is associated with pathogens of diverse grasses with agricultural significance. For example, *M. oryzae* includes pathogens of rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*),

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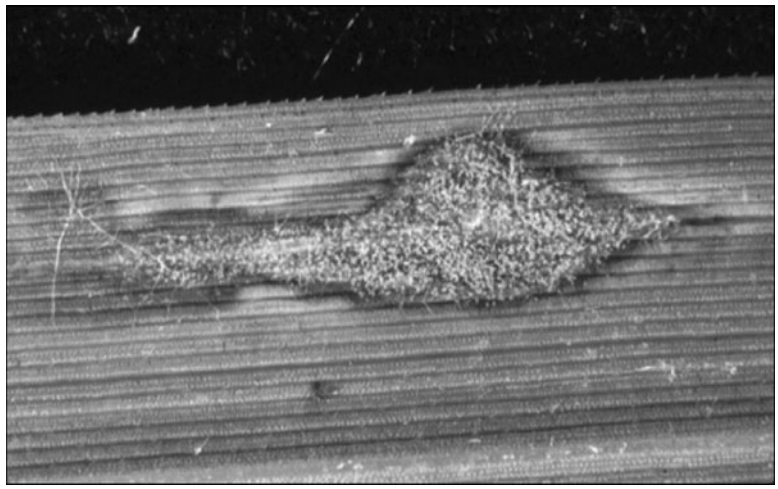

FIGURE 1 A sporulating leaf blast lesion on rice. Fully susceptible lesions first become visible ~5 days postinoculation (dpi) and mature ~7 dpi. Such lesions range from 0.5 to >1 cm in length depending on the rice variety and plant maturity in the field, and they produce several thousand conidia a day for about 2 weeks (Ou, 1985). (Photo courtesy of J. M. Bonman.)

oats (*Avena sativa*), finger millet (*Eleusine coracana*), Italian (foxtail) millet (*Setaria italica*), weeping lovegrass (*Eragrostis curvula*), and perennial ryegrass (*Lolium perenne*). This designation supported earlier conclusions of Kato and colleagues (Kato et al., 2000). They examined pathogenicity, mating compatibility, and restriction fragment length polymorphisms of *M. grisea* isolates from various hosts and found that isolates from *Oryza*, *Setaria*, *Panicum*, and *Eleusine* host species formed a genetically close, interfertile group (the crop isolate or CC group) that was distinct from *Pyricularia grisea* (Kato et al., 2000), the name first assigned to isolates from *Digitaria* host species. The *Triticum* and *Lolium* pathotypes were subsequently shown to belong to the *M. oryzae* (CC) group (Tosa et al., 2004).

Although in general, fungal isolates collected in nature are specialized for that host species, some isolates can infect other species as well (Urashima et al., 1993; Viji et al., 2001). Indeed, Heath and colleagues (Heath et al., 1990) presented comprehensive cytological analyses of a rice isolate and a finger millet isolate infecting rice, finger millet, and weeping lovegrass. They suggested that different plant-by-strain combinations represent a continuum of plant-fungus interactions. That is, isolates that were not adapted for a particular host could colonize that host at some infection sites, resulting in occasional small susceptible lesions or dark brown resistance spots (Valent et al., 1991). Studies of fully susceptible interactions, in which the fungus succeeds at most infection sites, require fungal isolates that are adapted for that host species.

Population Structure and Dynamics

The degree of sexual fertility exhibited by fungal isolates in different host-specific populations within the *M. grisea* species complex ranges from highly fertile hermaphrodites, to female-sterile strains that mate only with hermaphrodites, to totally infertile strains. Host range is generally predictive of fertility (Orbach et al., 1996). Most rice pathogens isolated from the field have low levels of fertility, being either female-sterile or totally infertile. In contrast, most isolates from finger millet or wheat are highly fertile hermaphrodites. Breeding of rice pathogens for improved fertility, as well as identification of rare hermaphroditic rice isolates, have made genetic analysis possible with rice pathogens (Wang and Valent, 2009).

Low levels of sexual fertility and geographic isolation of strains of opposite mating type suggested that field populations infecting rice were predominantly asexual. DNA fingerprinting using multilocus probes confirmed that rice isolates in younger rice-growing regions, such as the Americas, can be divided into relatively low numbers of distinct lineages, with each lineage apparently derived through clonal propagation from a common ancestor (Zeigler, 1998). However, such lineages were not apparent in the ancient Asian populations near the center of origin of rice. The occurrence of hermaphroditic rice isolates in these Asian populations is suggestive of some residual level of sexual recombination, although this has not been demonstrated. Analysis of blast lineages in South America suggested that individuals within lineages may have limited potential for overcoming some *R* genes, leading to the "lineage exclusion" strategy for predicting effective *R* gene combinations to control the disease (Zeigler, 1998; Wang and Valent, 2009).

Evolution of host-specific populations is an important topic that can be addressed within the *M. grisea* species complex. Based on 37 multilocus haplotypes among 497 fungal isolates, Couch and colleagues determined that the rice-infecting population had a single origin host shift from a *Setaria* population around the time of domestication of rice (Couch et al., 2005). That is, the current rice blast population dates from ~7,000 years ago, when cultivated rice (*O. sativa*) was domesticated from *Oryza rufipogon* in the middle Yangtze valley in China. Italian millet, *Setaria italica*, was domesticated in China around the same time. Additional shifts to rice weeds, *Panicum* and *Leersia* species, followed soon after. Additional host shifts might have occurred, especially around the time of the Green Revolution, ~35 years ago, when improved rice varieties spread rapidly over a broad geographic area. However, there is no evidence to support a second host shift event associated with rice. Host shifts probably account for the recent emergences of wheat blast in Brazil and GLS in the United States and Japan. Because rice blast was endemic to the region where wheat blast first occurred, early hypotheses focused on a

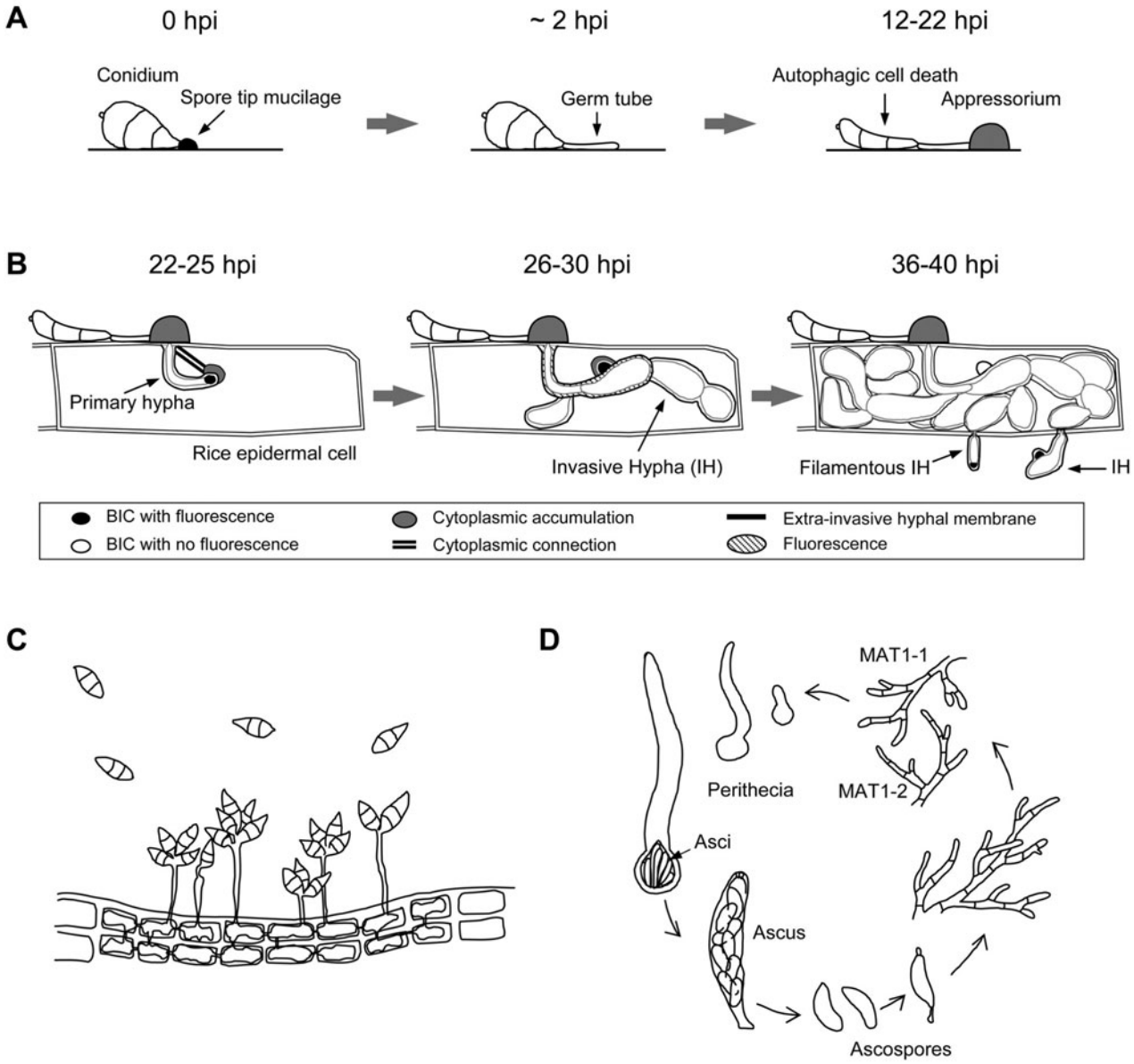

FIGURE 2 Asexual and sexual cycles of the rice blast fungus. (A) A conidium (25 to 30 by 9 to 12 μm) adheres to the leaf surface by using spore tip mucilage, produces a germ tube that senses the inductive surface, and differentiates an appressorium. A mature appressorium uses osmotically generated pressure to force a penetration peg through the plant cuticle and cell wall. (B) Inside the host cell lumen, the penetration peg becomes a filamentous primary hypha, accompanied by migration of cellular contents from the appressorium into the primary hypha. The primary hypha invaginates the host plasma membrane and secretes effectors, which are visualized by translational fusion of effector polypeptides with enhanced green fluorescent protein (GFP), into the membranous cap BIC at its tip at 22 to 25 h postinoculation (hpi). By 26 to 30 hpi, primary hyphae have differentiated into bulbous IH, which are sealed in an EIHM compartment. The BIC has moved beside the first differentiated IH, where it accumulates fluorescent effector proteins as long as IH grow in the cell. By 36 to 40 hpi, IH have undergone extreme constriction to cross the plant cell wall. In neighbor cells, the fungus first grows as filamentous IH secreting effector:GFP fusion proteins into tip BICs and then differentiates into bulbous IH with fluorescent side BICs. Subsequent cell invasions follow the same pattern. (C) Conidiogenesis in *M. oryzae* is holoblastic such that expansion and swelling of the conidiophore apex gives rise to a conidium, followed by a septum being formed to delimit the forming conidium (Howard 1994; Shi and Leung, 1995). The apex then grows to the side to produce the next conidium, resulting in three to five conidia borne sympodially on a conidiophore. (D) Sexual cycle: strains of opposite mating type mate to form pigmented perithecia (500 to 1,200 μm in length) with spherical bases (80 to 260 μm in diameter) and long cylindrical necks. Unordered asci contain eight hyaline, fusiform ascospores (16 to 25 by 4 to 8 μm), each with four cells and a single nucleus per cell.

host shift from rice to wheat. However, pathogenicity assays, the absence of rice pathogen-specific repetitive elements in the wheat isolates, and the extremely high sexual fertility of the wheat isolates compared to that of rice isolates proved that wheat pathogens were distinct from rice pathogens and that they were related to finger millet pathogens (Urashima et al., 1993). Interestingly, the wheat isolates from South America and GLS isolates from the United States and Japan are more closely related to each other than to other studied host-limited populations within the species complex (Viji et al., 2001; Farman, 2002a; Tosa et al., 2004). The growing intensities of wheat blast and GLS highlight the importance of understanding past host shifts and of determining the potential for new shifts to occur on additional crops.

Comparative Genomics

To facilitate understanding of *M. oryzae* infection strategies, the genome sequence was determined for strain 70-15, a fertile laboratory strain that is pathogenic on rice (Dean et al., 2005). Like most sexually fertile strains (Orbach et al., 1996), 70-15 has seven chromosomes. The assembled genome reported by Dean et al. (2005) contained 11,109 protein-coding genes, was 38.8 Mb in length, and was comprised of ~90% simple DNA sequence and ~10% repetitive DNA sequence. The currently available assembly 6, which is approaching a finished genome, has 11,074 predicted genes (http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/MultiHome.html). A community annotation database provides a valuable resource for analysis of *M. oryzae* genes (http://www.mgosdb.org/). The *M. oryzae* genome is enriched for putative secreted proteins, with estimates ranging from 7% (Dean et al., 2005) to 12% (Soanes et al., 2008) of all genes, depending on the prediction program used. Compared to the related pyrenomycete *Neurospora crassa*, expanded gene families in *M. oryzae* include many genes with a potential role in pathogenicity: genes encoding putative enzymes for degrading the plant cuticle and plant cell walls, genes for G-protein-coupled receptors (GPCRs) with potential roles in environmental sensing and response, and genes involved in production of secondary metabolites. The genome has expanded families for genes encoding small, secreted, cysteine-rich proteins. In other systems, these proteins often function as effectors, pathogen proteins secreted into host extracellular spaces or translocated into the cytoplasm of living host cells to control host defenses and cellular processes. From current experience, the subset of these effector genes, the avirulence (*AVR*) effector genes corresponding to rice *R* genes deployed by breeders in the field should exhibit increased polymorphism compared to genes not subject to *R*-gene-mediated selection. Comparing the 70-15 sequence with sequences from diverse rice pathogens should provide clues to identify AVR effectors.

Extensive expression data are available for fungal cell types, such as conidia, appressoria, perithecia, and mycelium from different culture conditions. These data include expressed sequence tags, serial analysis of gene expression, robust-long serial analysis of gene expression, massively parallel signature sequencing, and microarray analyses (Irie et al., 2003; Takano et al., 2003; Ebbole et al., 2004; Soanes and Talbot, 2005; Gowda et al., 2006). Whole *M. oryzae* genome microarray analysis has been performed for mycelium grown in nitrogen-rich and nitrogen-deficient media (Donofrio et al., 2006). In planta expression analyses have also been performed. Studies performed after macroscopic symptoms developed identified both fungal and rice genes expressed in planta (Kim et al., 2001; Rauyaree et al., 2001; Matsumura et al., 2003). Large-scale expressed sequence tag analysis (Jantasuriyarat et al., 2005) and microarray analysis (Vergne et al., 2007) performed at early infection stages before symptom appearance focused on rice gene expression because so little fungus was present in the infected leaf tissue. Mosquera et al. (2009) developed a robust procedure to obtain infected rice sheath tissue RNAs consisting of 20% RNA from biotrophic invasive hyphae (IH) growing in first-invaded cells, and they showed that these morphologically distinct IH specifically express many novel genes that encode putative secreted proteins.

Most repetitive sequences in *M. oryzae* are derived from transposable elements (Dean et al., 2005). Seven types of retrotransposons and three types of DNA transposons are prevalent in the 70-15 genome. These repetitive elements are not uniformly distributed in the genome but form discrete clusters. Analysis of these elements revealed that they have undergone extensive recombination during their evolution. These recombination events could cause deletions or inversions of the intervening sequences or chromosome translocations, which play important roles in genome evolution. Transposon-mediated recombination events may contribute to the *M. oryzae* genome instability that is characteristic of some subpopulations, including rice pathogens and perennial ryegrass pathogens. For example, most rice pathogens have lost sexual fertility and they have undergone extensive chromosome rearrangements (Talbot et al., 1993b; Orbach et al., 1996). Except for rare fertile strains, field isolates from rice often contain different numbers and sizes of chromosomes, even when they have similar molecular fingerprints. In addition, independent karyotype changes were found after prolonged serial transfer of one fungal strain in culture (Talbot et al., 1993b). This rice pathogen karyotype variability is associated with DNA deletions, inversions, and translocations. Genome instability of rice pathogens is also reflected by the rapid spontaneous mutation of certain genes involved in pathogenicity and host specificity, which tend to reside in transposon-rich regions of the genome. For example, rice pathogens undergo frequent spontaneous deletion of the *BUF1* melanin-biosynthesis gene (Valent et al., 1991), which is required for appressorial penetration. A genetic mechanism has been described for instability of certain *BUF1* alleles in sexual crosses (Farman, 2002b).

The abundance of transposable elements in the rice isolates from the field suggests that *M. oryzae* lacks the repeat-induced point mutation (RIP) mechanism described in the related pyrenomycete *N. crassa*. RIP is involved in detecting and mutating duplicated sequences during the sexual cycle. Dean et al. (2005) reported some low level of sequence signatures of RIP in some transposable elements in the 70-15 genome, but not in others, and concluded that RIP is not operating in *M. oryzae*. In contrast, Ikeda and colleagues (Ikeda et al., 2002) studied highly fertile wheat isolates from Brazil and concluded that a process similar to RIP functions in these strains. They reported that the frequency of transition mutations indicative of RIP correlated with the level of sexual fertility in the strains tested. This leads to the intriguing possibility that some host-specific populations within the *M. oryzae* species complex have different properties with regard to RIP as well as sexual fertility.

Another trait in which host-specific populations differ is telomere instability. Instability of telomere sequences in rice isolates has been documented, and indeed, many of the *AVR* genes identified are located near telomeres (Orbach et al., 2000; Farman, 2007). In contrast, telomeres from

foxtail millet and crabgrass isolates appear to be stable. Recently, it has been shown that ryegrass isolates responsible for GLS show unprecedented levels of telomere instability (Farman, 2007). Mechanisms and biological consequences for telomere instability traits in the host-specific field populations remain interesting subjects for analysis.

THE RICE BLAST DISEASE CYCLE: A NEW PARADIGM FOR HEMIBIOTROPHY

The rice blast pathogen undergoes extensive development to execute its disease cycle (Fig. 2A through C). *M. oryzae* was believed to be a hemibiotroph similar to *Colletotrichum* species, which invade one or a small number of host cells biotrophically and then undergo a distinct switch to necrotrophic killing of host cells before invading them (O'Connell and Panstruga, 2006). To investigate this, Berruyer et al. (2006) transformed a rice pathogen to express cytoplasmic enhanced yellow fluorescent protein (YFP) and used fluorescence stereomicroscopy to follow individual lesions from appressoria to macroscopically visible leaf spots. They found that the fungal growth front always preceded visible symptoms even at later stages, and asymptomatic infected regions were common. From these results and characteristics of sequential cell invasions in rice sheath epidermal cells, Kankanala et al. (2007) concluded that, at least in the highly susceptible interaction, there was not a distinct switch from biotrophy to necrotrophy as occurs for *Colletotrichum* species. Instead, rice blast defines a new paradigm in hemibiotrophy whereby each successive plant cell invasion is biotrophic but invaded plant cells die by the time the fungus moves to living neighbor cells (Kankanala et al., 2007). Many molecular and cellular details of the blast disease cycle have emerged (see also Talbot, 2003; and Ebbole, 2007).

Appressorium Formation and Function

Extensive structural and functional analyses have been reported for appressorium formation, which involves a series of developmental and metabolic processes (Bourett and Howard, 1990; Howard and Valent, 1996; Talbot, 2003; Ebbole, 2007). As shown in Fig. 2A, rice blast disease is initiated by conidia landing on the leaf surface and attaching tightly by virtue of a glue, called spore tip mucilage, that is released from the conidial apex upon hydration (Hamer et al., 1988). Conidia germinate to produce specialized germ tubes that recognize the physicochemical features of the surface they are contacting. Upon recognition of an inductive surface (one that promotes appressorium development), the germ tube ceases apical growth. The germ tube apex swells and hooks, and apical vesicles polarize toward the host surface (Bourett and Howard, 1990). The germ tube apex continues to enlarge and develops a symmetrical dome-shaped appressorium. A mitotic division occurs, and one of the daughter nuclei migrates into the developing appressorium (Bourett and Howard, 1990; Veneault-Fourrey et al., 2006). A septum forms between the appressorium and the remainder of the germling, leaving the appressorium as a separate unicellular structure with a single nucleus. At this point, appressorial melanization occurs. As the appressorium matures, it strongly adheres to the host cuticle and generates the highest turgor pressure known in any living organism, as high as 8.0 MPa. This pressure, which is equivalent to the pressure felt at 800 m under water or 40 times that of an automobile car tire, is translated into mechanical force to puncture the plant cuticle with a penetration peg (Howard et al., 1991; de Jong et al., 1997; Ebbole, 2007). Appressorium function demands an autophagic process, regulated by at least the *MgATG8* gene, to recycle the contents of the appressorium-bearing conidium before plant penetration (Veneault-Fourrey et al., 2006).

Appressorial development can be induced in vitro and on various artificial surfaces, including cellophane, Teflon, Mylar, and polyvinyl chloride, which has been very useful in identifying conditions, signal molecules, or genes that regulate appressorium development (Valent, 1997; Talbot, 2003; Ebbole, 2007). For instance, exogenous addition of chemicals, such as secondary messengers or their analogs (cyclic AMP [cAMP] or diacylglycerol [DAG]), or plant components (cutin or lipid monomers) induces appressorium development by germinating conidia on noninductive hydrophilic surfaces, whereas some other chemicals suppress the developmental process on inductive hydrophobic surfaces (Lee and Dean, 1993; Thines et al., 1997). Oh et al. (2008) have identified 357 genes, referred to as appressorium-consensus genes, which are differentially expressed during appressorium formation in vitro.

The cAMP signaling pathway in eukaryotes involves production of cAMP by an adenylate cyclase and activation of a protein kinase A (PKA) by cAMP binding to its regulatory subunit. This releases the catalytic subunit of PKA (CPKA) for phosphorylation of target proteins. Mutants with targeted deletion of the *Magnaporthe* adenylate cyclase (*MAC1*) gene fail to form appressoria (Choi and Dean, 1997). The defect was restored by addition of cAMP or by a mutation in a regulatory subunit gene of PKA (*SUM1*, for suppressor of the Mac1$^-$ phenotype) that causes constitutive activation of PKA (Choi and Dean, 1997; Adachi and Hamer, 1998). Mutants with deletion of *CPKA* still produce appressoria, although they are small and nonfunctional (Mitchell and Dean, 1995; Xu et al., 1997). On a noninductive surface, the CpkA$^-$ mutants respond to exogenous cAMP, suggesting the presence of an additional catalytic subunit of PKA (Xu et al., 1997). The *CPKA*/*SUM1*-encoded PKA regulates turgor generation in appressoria through rapid degradation of lipid and glycogen reserves (Thines et al., 2000). Direct measurement of cellular cAMP in germ tubes showed significantly higher accumulation of cAMP in the germ tubes growing on an inductive surface than on a noninductive surface (Liu et al., 2007a), confirming the role of cAMP in appressorium morphogenesis. How the fungus senses the nature of the contact surface and triggers cAMP signaling is currently unclear, but this might involve surface proteins secreted during appressorium formation. Mutants lacking MPG1 (a fungal hydrophobin) or CBP1 (a chitin-binding protein) are inefficient in producing appressoria (<10% of wild-type frequencies), but an increase of cellular cAMP restores appressorium formation in respective mutants, suggesting their roles upstream of the cAMP signaling pathway (Talbot et al., 1993a, 1996; Beckerman and Ebbole, 1996; Kamakura et al., 2002).

One of the expanded gene families in *M. oryzae* encodes GPCRs, transmembrane receptors in eukaryotes that sense extracellular signals and activate signal transduction pathways. In fungi, GPCRs are involved in the regulation of contact-dependent morphogenesis (Kulkarni et al., 2005; Kumamoto, 2008). *M. oryzae* has 76 GPCR-like proteins (Kulkarni et al., 2005), among which PTH11 is proposed to have a role in contact surface recognition (DeZwaan et al., 1999). Mutation of *PTH11* causes a defect in appressorium maturation but does not impair any other stages of the life cycle. Appressorium formation in the *pth11*$^-$ mutants is restored by addition of cAMP and DAG, indicating that PTH11 acts upstream of cAMP/PKA and DAG/protein

kinase C (PKC) signaling pathways. Heterotrimeric G proteins interact with GPCRs to relay signals to intracellular responders, such as adenylate cyclase, phospholipases, kinases, and ion channels (Li et al., 2007b). M. *oryzae* contains three Gα subunits (MagA, MagB, and MacC [Liu and Dean, 1997]), two Gβ subunits (Mgb1 and Mgb2 [Nishimura et al., 2003]), and one Gγ subunit (Liang et al., 2006). Among the Gα subunits, appressorium formation and virulence was impaired in *magB*$^-$ mutants but not in *magA*$^-$ and *magC*$^-$ deletion mutants (Liu and Dean, 1997; Fang and Dean, 2000). Disruption of the *magB* gene, however, caused pleiotropic effects, including defects in conidiation, sexual development, mycelial growth, and virulence, indicating a common involvement of G protein signaling in regulating cellular activities. All three Gα subunits interact with the regulator of G protein signaling, RGS1 (Liu et al., 2007a). Rgs1$^-$ mutants produce appressoria efficiently on both inductive and noninductive surfaces, which suggests that loss of RGS1 uncouples surface dependency during appressorial differentiation (Liu et al., 2007a). Similar to Rgs1$^-$ mutants, Rgs-insensitive or constitutively-active MagA strains accumulate high levels of cAMP and produce appressoria efficiently on inductive and noninductive surfaces. These results suggest a signaling pathway of MagA-dependent adenylate cyclase activation, resulting in an increase of cAMP.

The roles of mitogen-activated protein kinases (MAPKs) in fungal developmental processes and pathogenicity have been reviewed extensively (Xu, 2000; Zhao et al., 2007). In M. *oryzae*, three MAPKs, PMK1 (for pathogenicity MAPK), MPS1 (MAPK for penetration and sporulation), and OSM1 (for osmoregulation MAPK), are involved in infection-related development. PMK1, a functional homolog of the *Saccharomyces cerevisiae* FUS3/KSS1 kinases, regulates appressorium formation and is necessary for penetration and invasive growth (Xu and Hamer, 1996). PMK1 and two upstream components Mst7 (MAPK kinase) and Mst11 (MAPKK kinase) form the Mst11-Mst7-Pmk1 pathway. The adaptor protein Mst50 interacts with Mst11 and Mst7, and integrates diverse signals from Mgb1, Cdc42, Ras1, and Ras2 (Zhao et al., 2007). Activated PMK1 then moves into the nucleus and regulates target genes, including *MST12* (transcription factor) and *GAS1* and *GAS2* (encoding small proteins specifically expressed in appressoria) (Zhao et al., 2007). The PMK1 pathway also controls transfer of storage carbohydrates and lipids to the developing appressorium, where these storage reserves are rapidly degraded under control of the PKA pathway (Thines et al., 2000). M. *oryzae* MPS1 is required for appressorium function (Xu et al., 1998). MPS1 is a functional homolog of yeast SLT2, which regulates cell wall integrity. MPS1 appears to regulate cell wall remodeling during appressorium formation and polarity establishment required for producing a penetration peg. Mps1$^-$ mutants produce appressoria that are melanized but fail to penetrate and develop invasive hyphae. OSM1 appears to play a role in appressorium morphogenesis, but it is dispensable for plant infection (Dixon et al., 1999). OSM1 is a functional homolog of *S. cerevisiae* osmosensory MAPK HOG1 (for high-osmolarity glycerol) that regulates glycerol accumulation to maintain cellular turgor in response to hyperosmolarity. The *osm1*$^-$ mutants are sensitive to osmotic stress, but they do not have defects in glycerol accumulation and turgor generation in appressoria, or in plant infection, indicating that glycerol generation is not regulated by OSM1 in M. *oryzae*. Interestingly, *osm1*$^-$ mutants produce multiple appressoria under chronic hyperosmotic stress, suggesting a negative role of OSM1 in suppressing the PMK1 appressorium development pathway during stressful conditions.

Melanin and glycerol play key roles in the generation of hydrostatic pressure in the appressorium. Melanin is a gray pigment produced by polymerization of the polyketide precursor 1,8-dihydroxynaphthalene (DHN) and is deposited between the appressorial cell wall and membrane (Howard and Valent, 1996). The high turgor pressure in melanized appressoria is generated by solute molecules, including glycerol, that accumulate to high levels through metabolism of glycogen, trehalose, and lipids, which are the most abundant storage products in conidia (de Jong et al., 1997; Wang et al., 2005). Autophagy is a primary intracellular catabolic mechanism for degrading and recycling organelles and proteins during cellular development (Levine and Klionsky, 2004). Mutants lacking each of six autophagy-related genes (*MgATG1*, *-2*, *-4*, *-5*, *-9*, and *-18*), which were identified based on homology to corresponding *S. cerevisiae ATG* genes, failed to penetrate the host due to lower levels of appressorial turgor pressure, providing evidence that glycerol production in appressoria requires the turnover of cytoplasmic contents of conidia (Liu et al., 2007b). Nonmelanized appressoria fail to generate turgor pressure, due to the lack of efficient accumulation of glycerol, thus resulting in nonpathogenicity (Chumley and Valent, 1990; Howard et al., 1991; de Jong et al., 1997). Melanin biosynthesis genes *ALB1* (MGG_07219.5), *RSY1* (MGG_05059.5), and *BUF1* (MGG_02252.5), respectively named for their albino, rosy, and buff pigmentation in culture (Chumley and Valent, 1990), encode a polyketide synthetase, scytalone dehydratase, and trihydroxynaphthalene reductase, respectively. All three genes are highly induced during appressorium formation but dramatically repressed when the fungus enters the host cell and establishes biotrophic invasion (Oh et al., 2008; Mosquera et al., 2009). This suggests that melanin biosynthesis is required for penetration but not for biotrophic colonization. Genes for two putative transcription factors, *PIG1* (MGG_07215.5) and *HTF* (MGG_07218.5), are linked to *ALB1* (MGG_07219.5) and the *BUF1* homolog *4HNR* (MGG_07216.5) on chromosome 1 (Oh et al., 2008; Valdovinos-Ponce, 2007). Mutants lacking the PIG1 transcription factor fail to produce melanin in vegetative hyphae, but the appressoria are still melanized, suggesting that a different mechanism regulates appressorial melanization (Tsuji et al., 2000). Deletion of *HTF* does not cause a defect in melanization of mycelia or appressoria or a reduction in pathogenicity (Oh et al., 2008; Valdovinos-Ponce, 2007). Regulatory genes for appressorial melanization remain to be determined.

Although mechanical pressure appears to be the primary means of plant cuticle penetration, cell surface-modifying enzymes have been implicated in facilitating penetration (Howard et al., 1991; Skamnioti and Gurr, 2007). The cuticle is the outermost barrier of plants, and fungi secrete cutinases, serine esterases that hydrolyze cutin, the main component of plant cuticle (Kolattukudy, 1985). M. *oryzae* has 17 putative cutinase genes, which is the largest number found among all fungal genomes sequenced so far (Dean et al., 2005; Skamnioti et al., 2008). For example, the *Fusarium graminearum* and *N. crassa* genomes contain 12 and 3 cutinases, respectively. Unlike the *CUT1* gene (Sweigard et al., 1992), which is not differentially expressed during appressorial development, the *CUT2* gene (MGG_09100.5) shows dramatic upregulation during penetration (Skamnioti and Gurr, 2007). The *cut2*$^-$ mutant is much less pathogenic than the wild type due to a defect in penetration peg formation. Surface adhesion and appressorium turgor generation are unaffected in the

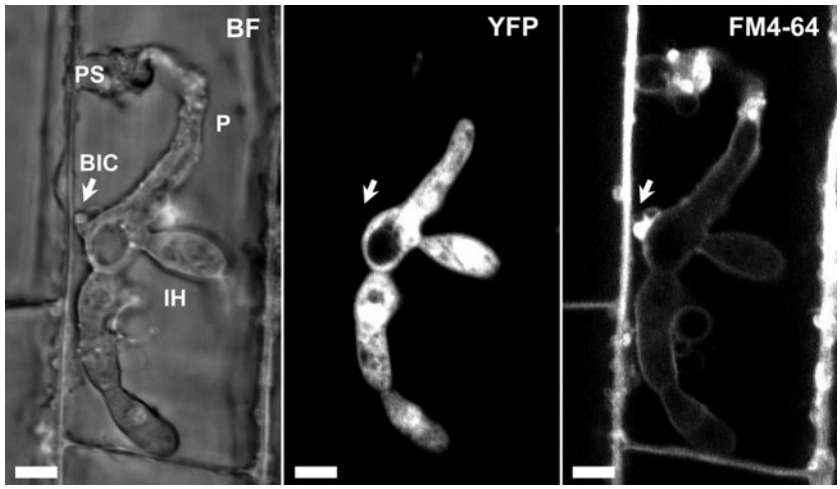

FIGURE 3 The EIHM tightly wraps the IH and prevents the endocytotic tracker dye FM4-64 from reaching IH membranes. At 29 hpi, an IH of a fungal transformant expressing cytoplasmic enhanced yellow fluorescent protein (YFP) is viewed by bright-field optics (left panel) and by YFP (middle panel) and FM4-64 (right panel) fluorescence (both shown as white). At this site, the primary hypha (P) extending from the appressorial penetration site (PS) had lost viability after IH formed (observed in ~50% of all infection sites). A BIC (arrow) beside the first IH cell is rich in FM4-64-stained membranes that are continuous with EIHM. Bars, 5 μm.

cut2^{-} mutant, providing direct evidence for a role for cutinase in penetration (Skamnioti and Gurr, 2007).

Biotrophic Invasive Hyphae Successively Colonize Live Rice Cells

Excellent cytological and ultrastructural analyses have been reported for the blast fungus interacting with host plant cells after penetration (reviewed by Kankanala et al., 2007). Once inside the epidermal cell, the penetration peg expands to form a narrow filamentous primary hypha that differentiates into thicker, bulbous IH in the compatible interaction (Heath et al., 1990). New details of biotrophic cell invasion have been described based on live-cell imaging in optically clear, excised rice leaf sheaths (Koga et al., 2004; Kankanala et al., 2007). Fungal and host cellular components were visualized with fluorescent dyes and reporter proteins. For example, studies with the endocytotic tracer dye FM4-64 answered the long-standing question of whether blast biotrophic hyphae breach the host plasma membrane to grow in direct contact with cytoplasm of the invaded cell or if they remain separated from the cytoplasm by invaginated plant plasma membrane (Kankanala et al., 2007). FM4-64 dye inserts into a cell's plasma membrane, moves by lateral diffusion in the membrane, and is actively internalized into the cell by the endocytotic pathway. The dye inserted into membranes of the rice sheath cells and into membranes of the primary hyphae, which appeared to invaginate the rice plasma membrane. In contrast, bulbous IH were precisely outlined by the dye (Fig. 3). The IH appeared to have been protected from FM4-64 insertion in their plasma membranes, presumably because they were sealed in plant membrane, named the extrainvasive-hyphal membrane (EIHM). Transmission electron microscopy of infected sheath samples, prepared by high-pressure freezing and freeze substitution to allow better membrane preservation, confirmed the presence of EIHM around IH. Apparently, differentiation of the primary hypha to IH included sealing the hyphae in an EIHM-bound compartment, where FM4-64 could not reach IH cellular membranes.

Live-cell imaging confirmed that sequential rice cell invasions were biotrophic, because invaded cells appeared to be healthy and plasmolyzed in sucrose solution, indicating an intact plasma membrane in the cell (Kankanala et al., 2007). Host cells failed to plasmolyze shortly before IH moved into neighbor cells, indicating that invaded host cells were no longer viable at that time.

Confocal imaging combined with time-lapse experiments demonstrated that IH appear to search along the plant cell wall for specific locations to cross into neighboring cells (Kankanala et al., 2007). Once found, the IH swell and send tiny penetration peg-like structures, the IH peg, across the cell wall (Fig. 2B). The extreme degree of constriction that IH undergo as they cross the plant cell wall is shown in Fig. 4. Additional transmission electron microscopy showing correlations of wall crossing points and pit fields with clustered plasmodesmata, failure of IH to enter guard cells that lack plasmodesmata, and additional observations strongly suggest that IH exploit plasmodesmata for movement into living neighbor cells (Kankanala et al., 2007).

Studies of in planta secretion of fluorescently labeled blast AVR effectors led to the discovery of a complex, pathogen-induced structure, the biotrophic interfacial complex (BIC), which accumulates these secreted proteins (R. Berruyer, C. H. Khang, P. Kankanala, S.-Y. Park, K. Czymmek, S. Kang, and B. Valent, unpublished data). The experiments involved the AVR-Pita1, PWL1, and PWL2 effectors, which are predicted to be translocated into the cytoplasm of living rice cells. Effector secretion was observed in planta using fungal transformants that expressed translational fusions of various fluorescent proteins at the C termini of different effector polypeptides under control of their native promoters. This analysis showed that BICs develop in two stages coupled to differentiation of intracellular filamentous hyphae into IH (Fig. 2). First, fluorescent effector proteins were secreted into the dome-shaped "membranous caps," extensions of the EIHM at the tips of primary hyphae in first-invaded cells (Fig. 2B, 22 to 25 h postinoculation [hpi]) and of filamentous IH in subsequently invaded cells (Fig. 2B, 36 to 40 hpi).

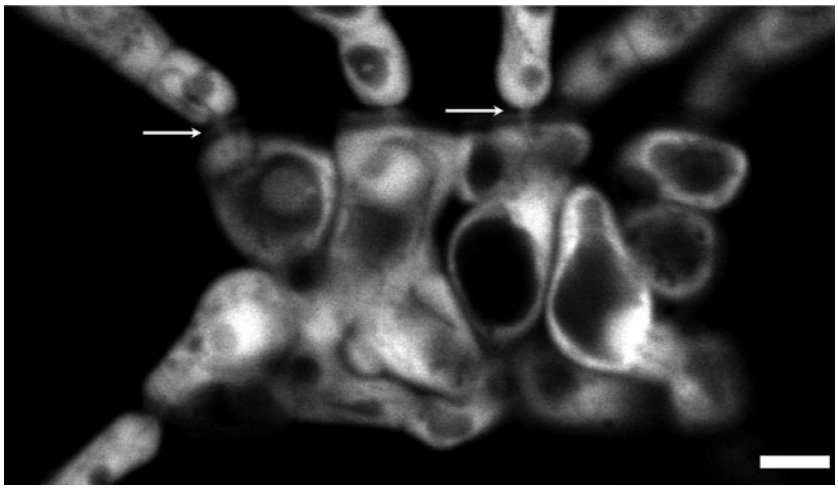

FIGURE 4 IH exhibit extreme constriction as they cross the rice cell wall at 32 hpi. YFP fluorescence in the fungal cytoplasm (in white) is shown alone to highlight the constriction (arrows). Bar, 5 μm. Reproduced with permission from Kankanala et al., 2007.

When these filamentous hyphae first swelled into IH, fluorescent EIHM caps moved to form a visible structure beside the differentiating IH cell (Fig. 2B, 26 to 30 hpi and 36-40 hpi). Fluorescent effectors remained localized to the BIC region as long as IH continued to grow in the rice cell (Fig. 2B). Secreted effector fusions partially colocalized with an aggregation of plant endocytotic membranes that labeled with FM4-64 (Fig. 3, arrows). Host cytoplasmic dynamics were focused around both the membranous cap- and IH-BICs during the early hyphal differentiation stage. Correlative light and electron microscopy showed that BICs corresponded to the complex aggregations of lamellar membranes and diverse vesicles previously reported between the IH cell wall and EIHM (Kankanala et al., 2007). Accumulation of secreted effectors and dynamic connections with host cytoplasm suggested that BICs are a hub of communication between IH and the host cell, with a likely role in effector secretion into the host cytoplasm.

Other than *AVR* effector genes and a nucleus-localized protein, MIR1 (Li et al., 2007a), genes that were specifically expressed by biotrophic IH have not been identified. Identification of fungal and rice genes expressed during biotrophic invasion in leaves has been difficult because so few host cells have encountered the pathogen at early infection stages (Berruyer et al., 2006). After enriching for IH RNA (up to 20% of the total) in infected rice sheath tissue, Mosquera et al. (2009) identified many genes that were highly upregulated in IH, including many encoding novel biotrophy-associated secreted (BAS) proteins that are excellent candidate effectors. Functional analyses of these candidate effector genes and of the host "effector-triggered-susceptibility" genes will begin to associate molecular mechanisms with the cell biology of biotrophic invasion of rice.

Necrotrophic Growth and Sporulation

Necrotrophic hyphae in blast disease presumably follow invasion by biotrophic IH (Berruyer et al., 2006), perhaps around the time that visible symptoms occur, in order to utilize host cell walls and other nutrients before sporulation. Extensive evidence suggests that necrotrophic hyphae do play a role in tissue colonization. Cytological studies have shown extensive destruction of mesophyll cells in some fungus-plant interactions (Heath et al., 1990; Rodrigues et al., 2003). The *M. oryzae* genome contains many genes that encode known and hypothetical plant cell wall-degrading enzymes, and these genes belong to expanded gene families (Dean et al., 2005). For example, there are 20 putative xylanase encoding genes in the *M. oryzae* genome (Wu et al., 2006). Mutants lacking individual *XYL1*, *XYL2*, *XYL6*, or both *XYL1* and *XYL2* genes were as pathogenic as the wild type, indicating that they are not individually required for pathogenicity. How these enzymes contribute when the fungus infects plant tissues remains to be determined (Wu et al., 1997, 2006).

The rice blast fungus produces a variety of phytotoxic metabolites, including tenuazonic acid (TA), picolinic acid, piricularine, pyriculol, pyriculariol, coumarin, tyrosol, and 9,12-octadecadienoic acid (Valent and Chumley, 1991; Park et al., 2008), which have been associated with blast lesion development. When dropped on a leaf surface, TA induces local necrosis resembling blast lesions, and TA-deficient mutants produce fewer lesions than the wild-type strain (Aver'yanov et al., 2007). The blast fungus, strictly known as a monocot pathogen in the field, was recently shown to infect certain ecotypes of *Arabidopsis thaliana*, a dicot model plant (Park et al., 2008). Unlike hemibiotrophism on rice and other grass hosts, infection of *A. thaliana* appeared to be necrotrophic and occurred with the aid of phytotoxic metabolites, including 9,12-octadecadienoic acid. Some of the blast fungal metabolites, such as TA and picolinic acid, were demonstrated to be hypersensitive-response elicitors, inducing resistance responses in rice (Zhang et al., 2004; Aver'yanov et al., 2007). Further studies are needed to identify the biosynthetic pathways for these metabolites and to understand their roles in contributing to pathogenicity or to resistance responses.

PKS-NRPS, hybrids of polyketide synthases and nonribosomal peptide synthetases, are newly discovered fungal enzymes involved in secondary metabolite biosynthesis. *M. oryzae* contains 9 PKS-NRPS genes (Collemare et al., 2008). Appressorium-specific expression of *ACE1* (the *AVR* gene described below), *SYN2*, and *SYN8* suggests that the resulting metabolites play a role during penetration, possibly by inhibiting defense responses of the epidermal cells. *SYN6* was also suggested to play a role in colonization. Single-deletion mutants lacking *ACE1*, *SYN2*, and *SYN6* were as pathogenic as the wild type on susceptible rice, suggesting that

the corresponding metabolites are not required for infection or that functionally redundant metabolites are produced.

The rice blast fungus produces elliptical lesions with grey centers (from abundant conidiophores) under sporulating conditions of high humidity (Fig. 1). Conidiation requires a period of darkness as a cue for conidial development, regulated by a blue-light receptor, mgwc-1 (MG03538.4; ortholog of *N. crassa* white collar-1), and mature conidia are released in the dark (Barksdale and Asai, 1961; Lee et al., 2006). Previous genetic studies have identified several loci (e.g., *CON*, *ACR*, and *SMO*) that control conidiation and conidial morphology (Hamer et al., 1989; Shi and Leung, 1995; Lau and Hamer, 1998). Strains with mutations of *CON5* and *CON6* fail to produce conidia. *CON5* was found to be epistatic to CON6 because the *con5*$^-$ mutant produces no conidiophores, but the *con6*$^-$ mutant produces conidiophores that do not bear conidia (Shi and Leung, 1995). Four other genes, *CON1*, *CON2*, *CON4*, and *CON7*, appear to act downstream from *CON5* and *CON6*, because these mutants show defects in conidiation (Shi and Leung, 1995). CON7 encodes a transcription factor regulating expression of genes involved in morphogenesis, including the GPCR gene *PTH11* and genes involved in cell wall formation (Odenbach et al., 2007).

Mutations that affect conidiation are often associated with defects in appressorium formation and pathogenicity, as observed in a variety of mutants, including Con$^-$, Acr1$^-$, Smo$^-$, Cut2$^-$, MagB$^-$ (Hamer et al., 1989; Shi and Leung, 1995; Liu and Dean, 1997; Lau and Hamer, 1998; Liu et al., 2007a; Skamnioti and Gurr, 2007), Mac1$^-$ (Choi and Dean, 1997), and Mps1$^-$ (Xu et al., 1998) mutants. This can be explained, at least partly, by the fact that conidiation and infection structure differentiation in fungi are regulated by G-protein-mediated signaling (Li et al., 2007b). In *M. oryzae*, Rgs1 and MagB in fact control conidiation (Liu et al., 2007a). Overexpression of Rgs1 inhibits conidiation, and the deletion of Rgs1 leads to hyperconidiation. Consistent with this, RGS-insensitive MagB also shows hyperconidiation.

Jeon et al. (2007) analyzed 21,070 transferred-DNA-tagged mutants by using a high-throughput screening system for seven phenotypes. Many of the mutants that are impaired in pathogenicity (559 mutants) are also impaired in asexual development, i.e., conidiation and conidial morphology (207 mutants; 37%), and 66% of the latter mutants are also defective in appressorium formation. This study identified a putative GTPase (MGG_02731; RAC homolog from *Colletotrichum trifolii*) and a protein involved in protein trafficking through the Golgi network (MGG_02423; ERD2 homolog from *S. cerevisiae*) that play roles in asexual development.

HOST SPECIFICITY IN RICE BLAST DISEASE

Effector Action in Pathogenicity and Resistance

Blast effector proteins are presumed to be delivered to the cytoplasm of living host cells to control host defenses and promote disease. *AVR* genes, whose products trigger hypersensitive resistance in invaded cells, encode effectors that happen to be recognized by corresponding *R* genes. Map-based cloning strategies based on measuring this AVR activity resulted in cloning of four *AVR* genes (Ebbole, 2007). Two of these are *AVR-CO39*, encoding a small polypeptide (Farman et al., 2002), and *ACE1*, encoding a PKS with a C-terminal NRPS that is expressed only in appressoria (Böhnert et al., 2004). The *PWL* genes are *AVR* genes that confer host species specificity by preventing the fungus from infecting weeping lovegrass (Kang et al., 1995; Sweigard et al., 1995). The *PWL2* gene occurs in rice-infecting strains, and it encodes a 145-amino-acid, secreted, glycine-rich, hydrophilic protein. *PWL1* encodes the *PWL2* ortholog from finger millet-infecting strains, and it encodes a 147-amino-acid secreted protein with 75% amino acid identity to PWL2 protein. *AVR-Pita1* stands out as the strongest candidate for a fungal effector gene. *AVR-Pita1* (Orbach et al., 2000; Khang et al., 2008), the *AVR* gene corresponding to the blast resistance gene *Pi-ta* (Bryan et al., 2000), encodes a putative zinc metalloprotease that is specifically expressed by biotrophic hyphae in planta. In yeast two-hybrid assays and in in vitro membrane binding assays, the mature protease binds specifically to the leucine-rich domain at the C terminus of the Pi-ta resistance protein (Jia et al., 2000). A rice biolistic transient expression assay indicated that the mature AVR-Pita1 metalloprotease functions in rice cells to trigger Pi-ta-mediated hypersensitive resistance (Jia et al., 2000). These results, together with the probable localization of *Pi-ta* in the plant cytoplasm, suggest that the blast fungus delivers the mature protease inside rice cells. How AVR-Pita1 functions as an effector inside the host cells remains to be discovered.

It is important to understand how the fungus so quickly defeats *R* genes deployed for disease control. In laboratory studies, some blast *AVR* genes undergo frequent spontaneous mutations and deletions due to flanking repetitive sequences and to location in the unstable regions near telomeres (Sweigard et al., 1995; Orbach et al., 2000). Examples of spontaneous transposon-mediated insertional mutation events that inactivated *AVR* genes and changed the cultivar specificity of rice blast strains have been documented for *AVR-Pita1* in laboratory studies (Kang et al., 2001) and for *AVR-Pita1* and *ACE1* in the field (Fudal et al., 2005; Zhou et al., 2007). Clearly, the abundant repetitive sequences in *M. oryzae* contribute to rapid genome evolution, and the resulting loss of gene function can change host cultivar specificity.

Although there is no direct evidence, circumstantial evidence suggests that AVR-Pita and PWL effectors function in pathogenicity for hosts lacking corresponding *R* genes (Kang et al., 1995; Sweigard et al., 1995, Khang et al., 2008). The *AVR-Pita* and *PWL* genes are members of multigene families that are widely distributed among isolates from diverse host species, and some members lack AVR activity, suggesting functional divergence as well as positive selection for maintenance in pathogen populations. The presence of rice-specific functional AVR-Pita members among strains nonpathogenic to rice also suggests they have roles during infection. In planta specific expression of *AVR-Pita* and *PWL* genes also supports a role in pathogenicity.

Host Species Specificity

The recent emergence of wheat blast and GLS as serious disease problems highlights the importance of understanding the molecular and mechanistic bases of host species specificity in the *M. grisea* species complex. Multiple examples of single genes that confer host species specificity have been described since the original report by Yaegashi in 1978 (Yaegashi, 1978). These include *PWL1* from finger millet pathogens and *PWL2* from rice pathogens, which prevent these strains from infecting weeping lovegrass (Kang et al., 1995; Sweigard et al., 1995). Tosa and colleagues crossed a wheat isolate with a *Setaria* isolate, an oat isolate, and a rice isolate and identified five loci that impact pathogenicity toward wheat. The *PWT1* and *PWT2* genes were identified from both rice and *Setaria* pathogens (Murakami et al., 2000;

Tosa et al., 2006), and the *PWT3* and *PWT4* genes were identified from the oat pathogen (Takabayashi et al., 2002). A fifth gene, *PWT5*, was identified only in the rice pathogen (Tosa et al., 2006). Interestingly, these genes induced different physiological responses in wheat; *PWT1* and *PWT5* induced a hypersensitive response, and *PWT2* induced papillae that block fungal entry into the host tissue. The *PWT3* gene from the oat isolate appeared to be a temperature-sensitive *AVR* gene (Takabayashi et al., 2002). The other gene, *PWT4*, exhibited wheat cultivar specificity, and identification of a corresponding wheat *R* gene confirmed a gene-for-gene relationship in wheat blast (Takabayashi et al., 2002). Similar results were reported for specificity towards *Setaria* spp. (Murakami et al., 2003). An independent study identified two *AVR* genes from a *Digitaria* isolate, *PRE1* and *AVR2*, which control rice cultivar specificity (Chen et al., 2006). Not all differences in host species specificity are due to *AVR*-like genes. One study showed polygenic segregation of genes from a finger millet pathogen controlling the extent of lesion development on rice, as well as *AVR* genes corresponding to different rice cultivars (Valent et al., 1991). However, *AVR* genes are clearly associated with host species specificity. The simple genetic differences between species-adapted isolates suggested that these strains are genetically close and that they could evolve to infect different grass species.

Effector Secretion

Biotrophic or hemibiotrophic fungi must deliver effector proteins across plant cell membranes into living plant cells in order to block defenses and control cell metabolism. This process is not understood for any fungus, even though extensive research is focused on the type III secretion system used by bacteria for delivering effectors inside host cells (Alfano and Collmer, 2004) and on the roles for protein translocation motifs (RXLR) in delivering oomycete effectors into the host cytoplasm (Morgan and Kamoun, 2007). For rice blast disease, the increasing numbers of *AVR*-like genes that control host specificity and the large number of R proteins that are predicted to be localized in the rice cytoplasm are consistent with the hypothesis that *M. oryzae* translocates many effectors into the host cytoplasm. With the exception of ACE1, known effector proteins have N-terminal signal peptides for entry into the endoplasmic reticulum (ER)-mediated secretion pathway. Involvement of ER-mediated secretion was confirmed by Yi et al. (2009) when they demonstrated that LHS1, the ER-localized heat shock protein 70 (HSP70) chaperone with an important role in ER import and protein folding, is required for effector secretion. Differential accumulation of known effectors (Berruyer et al., unpublished) and some BAS proteins (Mosquera et al., 2009) in BICs at the hypha-rice cell interface, together with characterization of Golgi-localized APT2 with a role in effector secretion (Gilbert et al., 2006), suggested that the effector secretion pathway might diverge after entry into the ER. The disproportionate need for proper protein secretion during biotrophic tissue colonization, demonstrated with *lhs1*$^{-}$ mutants (Yi et al., 2009), shows the importance of understanding protein secretion mechanisms in *M. oryzae*.

After secretion from the fungus, a subset of BAS proteins colocalize with effectors in BICs (Mosquera et al., 2009), supporting a hypothesized role for BICs in translocation of effectors into the host cytoplasm. Recent results showed that indeed, fluorescent effector proteins that accumulate in BICs are delivered to the cytoplasm of invaded cells (C. H. Khang and B. Valent, unpublished results). Interestingly, these fluorescent effectors also enter neighboring cells that are not yet invaded by the fungus. The secreted protein BAS4, which accumulates uniformly throughout the interfacial matrix surrounding IH, was not translocated to the rice cytoplasm. Thus, the way is open to understand effector translocation mechanisms, to identify fungal translocation motifs, and to identify the set of effectors in the *M. oryzae* genome.

FUTURE PROSPECTS

The rice blast system offers unique opportunity for basic research on fungal hyphal development and host interactions impacting a major plant disease and for applied research on developing effective strategies for achieving durable resistance. Towards these goals, major efforts must go into identifying the entire sets of effectors required for disease, into understanding how these effectors are delivered into living host cells, and into defining effector functions in promoting disease or triggering resistance. These studies are now being facilitated by the growing blast genome and cell biological resources. Great progress has been made in understanding pathogenicity mechanisms, especially before penetration into the host tissue, but much remains to be learned. For example, although signal transduction pathways are relatively well understood, little is known about the initiating signaling cues or the downstream morphogenetic responses. Interesting questions concern how the fungus executes its amazing biology associated with appressorial penetration pegs breaching the tough plant surface and then gently colonizing living cells, and associated with IH pegs, perhaps co-opting plasmodesmata to enter living neighbor cells. Much remains to be learned in understanding biotrophic invasion, the switch to necrotrophic growth, and sporulation.

The *M. grisea* species complex is a thriving collection of host plant-adapted populations. The rice-infecting population has so far eluded decades of effort on sustainable control strategies, and the recently emerged diseases on wheat and on perennial ryegrass appear to be just as difficult to control. In addition to understanding mechanisms of rice cultivar specificity, the *M. grisea* species complex presents exciting opportunities for understanding the mechanisms of host species specificity and fungal population shifts to specialize on new plant species. Increasing evidence suggests that, like rice cultivar specificity, host species specificity is often due to *R*-gene-mediated recognition of blast effector proteins. Comparative genomics should help identify effector sets that confer host species specificity as well as host cultivar specificity. Understanding how *AVR* effector genes are evolving in the field holds great potential for discovering novel routes to durable disease resistance.

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Fusarium Genetics and Pathogenicity

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INTRODUCTION

The genus *Fusarium* is one of the most extensively studied of all fungi. Various members of the genus are associated with plant diseases and the production of various classes of mycotoxins, with many secondary metabolites still only poorly characterized (Desjardins, 2006). In terms of direct infections of domesticated animals and humans, the number of reports is smaller. Human infections often are serious, e.g., causing keratitis (Foroozan et al., 2000; Hemo et al., 1989; Pagliarusco et al., 1995; Rosa et al., 1994) or infecting wounds (Leu et al., 1995; Tomimori-Yamashita et al., 2002; van Dijk et al., 1980), and are difficult to treat due to the lack of effective anti-*Fusarium* agents. Systemic infections, especially of immunocompromised individuals, usually are all but impossible to treat successfully and often result in death (Eljaschewitsch et al., 1996; Guarro et al., 2000; Mselle, 1999).

Taxonomy

Fungi within the genus *Fusarium* have long been studied in detail by taxonomists and plant pathologists attempting to identify and distinguish the causal agents of numerous plant diseases. Over 80% of the economic plants with disease lists maintained by the American Phytopathological Society have at least one disease incited by a *Fusarium* species (www.apsnet.org/online/common/search.asp). The genus has served as a taxonomic clinic almost since its first description in 1809 (Link, 1809). The most important morphological characters usually are the shape and means of production of the asexual spores, i.e., the macro- and microconidia (Fig. 1). By 1935, there were >1,000 identified taxonomic entities within the genus, which Wollenweber and Reinking (1935) reduced to 16 sections, 65 species, and 77 additional subspecific varieties and forms. Many of the species defined by Wollenweber and Reinking remain in use today. In the 1940s and 1950s, Snyder and Hansen (1940, 1941, 1945, 1954) reduced the number of recognized species to nine. This reduction has largely been undone, although there are two notable exceptions, *F. solani* and *F. oxysporum*, each of which contains a plethora of as-yet-undistinguished species and numerous strains of plant pathogenic importance that remain as they were designated by Snyder and Hansen. Species such as *F. moniliforme* and *F. roseum* contained so many different species that older literature that uses these names is problematic unless the cultures are available for inspection and evaluation. Correct identification remains a difficult task, even with molecular techniques, as the distinction between species and populations may be difficult to discern (Leslie and Summerell, 2006).

Genetic studies have helped define a number of species and have laid the groundwork for more-detailed molecular and physiological analysis. The biological species concept was first introduced by Hsieh et al. (1977) regarding *F. moniliforme* and by Matuo and Snyder (1973) regarding *F. solani* with different biological species usually termed "mating populations." For *F. moniliforme*, Leslie (1991) broadened the concept and developed the first female-fertile tester strains to be used for diagnostic purposes. These strains are now generally available through the Fungal Genetics Stock Center (Department of Biological Sciences, University of Missouri—Kansas City). The mating-population terminology has persisted but has become less common as many of these mating populations have now been given distinct species names. Species based on the biological species concept have generally been well accepted, although rare cases of cross hybridization between different biological species are known (Leslie et al., 2004).

The application of phylogenetic species concepts have been more controversial. Within the species derived from the old *F. moniliforme*, many of these newly defined species have been accepted (Nirenberg and O'Donnell, 1998). The proposed split of *F. graminearum* into numerous species (O'Donnell et al., 2004) has not been as well accepted and appears to be inconsistent with both the biological and morphological species concepts (Leslie and Bowden, 2008).

Dispersion and Reproduction

Fusarium spp. use a number of techniques to persist and disperse under field conditions. Virtually all strains produce asexual spores, with macroconidia and microconidia the

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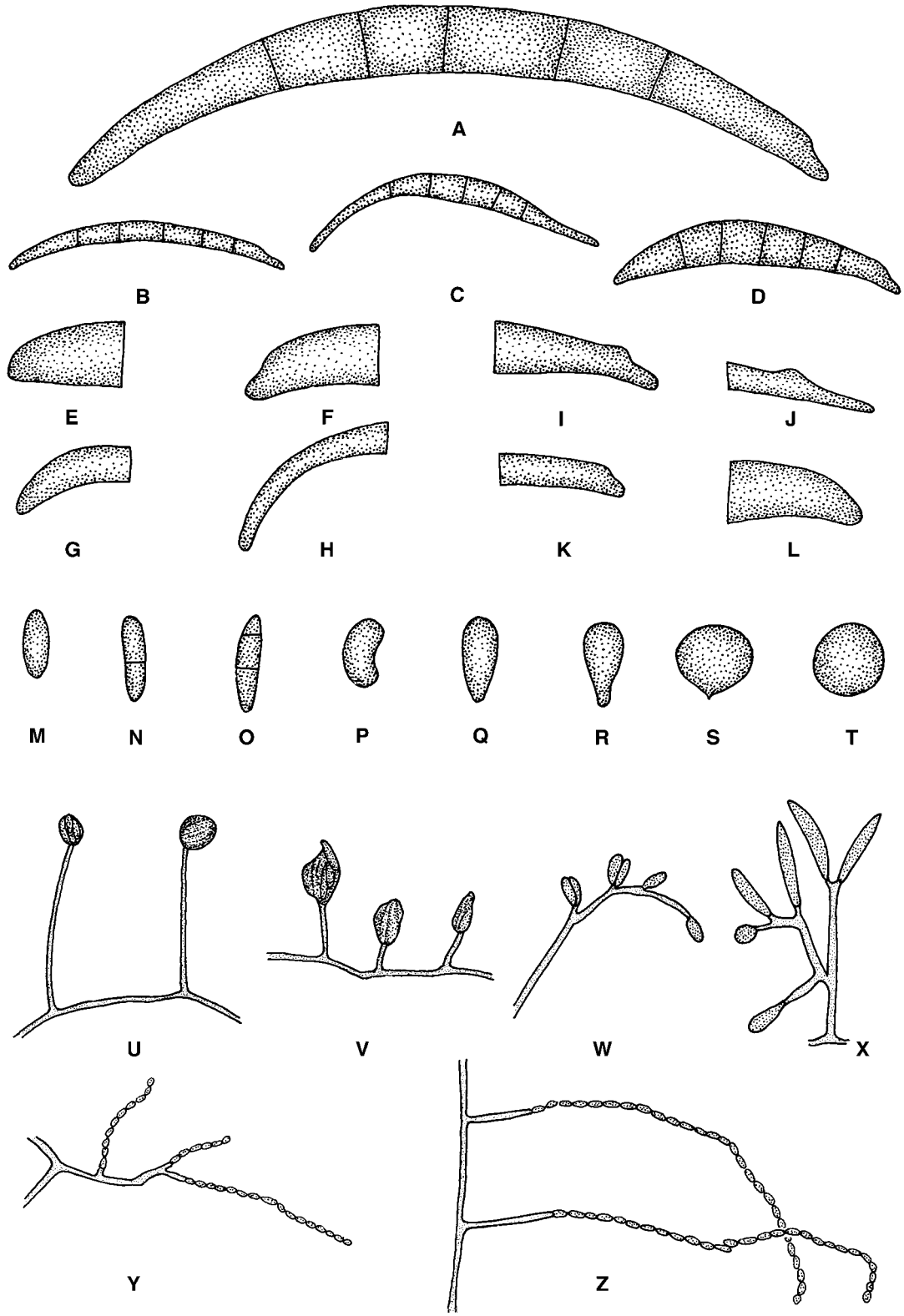
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most common such spore types and mesoconidia much less frequent. Members of different species may produce these spore types at different relative frequencies (including 0 and 100%). Furthermore, the presence or absence of one or more of these spore types, their shape, and their method of production are important species-diagnostic characters (Leslie and Summerell, 2006). These asexual spores may be wind or splash dispersed. In their free form, asexual spores are probably not viable for an extended period of time, as they are hyaline, UV sensitive, and frequently relatively thin walled. In the field, they may persist inside plants, in protected locations on soil, or on plant surfaces. Under laboratory conditions, spore survival varies by species, with members of *F. verticillioides* and related species possessing the best survival and members of *F. graminearum* and related species exhibiting the poorest survival. Strains can be maintained for several years, and in some cases indefinitely, by freezing spores at −80°C in 15 to 25% glycerol (vol/vol), by drying spores onto silica gel and preserving dry at 4°C, by lyophilizing cultures growing on carnation leaves, or as cultures dried on filter paper (Windels et al., 1988, 1993).

Species with a sexual stage produce haploid ascospores in a perithecium (Fig. 2). Ascospores appear to be very important for some diseases (e.g., head scab of wheat) and of little or no pathogenic importance in other diseases (e.g., ear and stalk rot of maize). In some cases (e.g., *F. graminearum*), perithecia are easy to identify and manipulate. In other cases (e.g., *F. verticillioides)*, perithecia may form readily in culture if the proper strains and environmental conditions are used, even though the sexual stage is observed at most rarely under field conditions. Finally, there are some species (e.g., *F. oxysporum*) that appear to reproduce asexually exclusively. Some species are presumed to have a sexual stage, even though perithecia associated with the species have not been observed from field material or in laboratory culture.

Most species are typical heterothallic ascomycetes that rely on widely conserved mating-type idiomorphs (Hornok et al., 2007) to regulate the mating-type process. At least one homothallic species is known (*F. graminearum*), but no pseudohomothallic species have been described. The importance of asexual conidia for reproduction, dispersal, and colonization is emphasized by the relative lack of strains that can serve as female parents in crosses in most field populations of pathogenic *Fusarium* spp. (Leslie and Klein, 1996). Thus, for most *Fusarium*-induced diseases asexual spores are probably a much more important source of inoculum than are the sexually produced ascospores. The long-term viability of ascospores has been the subject of debate. These spores are hyaline and desiccate relatively easily, yet they also have been recovered at significant heights (Schmale et al., 2006) and may be effective long-distance dispersal agents (Zeller et al., 2004).

Chlamydospores (Fig. 3) are produced by many species commonly recovered from soil. These spores are very effective for long-term survival under harsh environmental conditions, although the conditions required to germinate these spores have not always been clearly identified. The presence of chlamydospores is an important diagnostic character for some species, but the trait is not evolutionarily well conserved. Chlamydospores themselves are not thought to have a role in pathogenicity processes other than to ensure that vegetative hyphae and/or spores are available to colonize a host when a host becomes available.

The ability of many *Fusarium* spp. to persist as endophytes within apparently healthy plants is another important survival and dispersal mechanism. Asymptomatic growth within the host provides the fungus with a consistent and fairly defined source of nutrients and some protection against environmental extremes. Endophytic growth also enables relatively safe dispersion over long distances in plants or plant parts, e.g., seeds, without exposure of the fungus to adverse environmental conditions, e.g., UV light. As an endophyte, the fungus also can effectively colonize the entire plant with relatively minimal competition. The benefits of such systemic colonization are clear for the fungus, but the benefits for the plant are not as well understood. Under stress conditions, or when an appropriate stage of the plant's life cycle is reached, the fungus changes to a pathogenic lifestyle and induces disease in the plant. The specific nature of these presumably physiological signals is largely unknown, although changes in the carbon/nitrogen ratios within the plant and differential regulation of the utilization of some nitrogen and carbon sources have both been suggested as possibilities. Identifying and developing novel ways to prevent the internal shift from endophyte to pathogen could be important in reducing the damage caused not only by *Fusarium* spp. but also by other filamentous fungi that have a similar stage in their life cycle. Given the genetic and molecular tools available in many important pathogenic *Fusarium* spp., one of these pathosystems (e.g., *F. verticillioides* on maize) could become an important model system. A similar understanding of the conditions that trigger in planta production of various secondary metabolites could be of equal economic importance in reducing preharvest mycotoxin contamination.

GENERAL GENETICS

Complete genome sequences and other genomic level data (Table 1) are available for several species, including *F. verticillioides*, *F. graminearum* (two strains), *F. oxysporum*, and *F. solani*. In addition, detailed physical and recombination-based genetic maps are available for *F. verticillioides* (Jurgenson et al., 2002a; Xu and Leslie, 1996) and *F. graminearum* (Gale et al., 2005; Jurgenson et al., 2002b; Lee et al., 2008),

FIGURE 1 Spore morphology characters used in the identification of *Fusarium* species. Drawings are idealized and not necessarily to scale. (A through D) Macroconidial shapes. (A) Typical *Fusarium* macroconidium; the apical cell is on the left, and the basal cell is on the right; (B) slender, straight, almost needle-like macroconidium, e.g., *F. avenaceum*; (C) macroconidium with dorsoventral curvature, e.g., *F. equiseti*; (D) macroconidium with the dorsal side more curved than the ventral, e.g., *F. crookwellense*. (E through H) Macroconidial apical cell shapes; (E) blunt, e.g., *F. culmorum*; (F) papillate, e.g., *F. sambucinum*; (G) hooked, e.g., *F. lateritium*; (H) tapering, e.g., *F. equiseti*. (I through L) Macroconidial basal cell shapes; (I) Foot shaped, e.g., *F. crookwellense*; (J) elongated foot shape, e.g., *F. longipes*; (K) distinctly notched, e.g., *F. avenaceum*; (L) barely notched, e.g., *F. solani*. (M through T) Microconidial spore shapes; (M) oval; (N) two-celled oval; (O) three-celled oval; (P) reniform; (Q) obovoid with a truncate base; (R) pyriform; (S) napiform; (T) globose. (U through X) Phialide morphology; (U) monophialides, e.g., *F. solani;* (V) monophialides, e.g., *F. oxysporum*; (W) Polyphialides, e.g., *F. polyphialidicum*; (X) polyphialides, e.g., *F. semitectum*. (Y and Z) Microconidial chains; (Y) short chains, e.g., *F. nygamai*; (Z) long chains, e.g., *F. verticillioides*. (After Leslie and Summerell, 2006.)

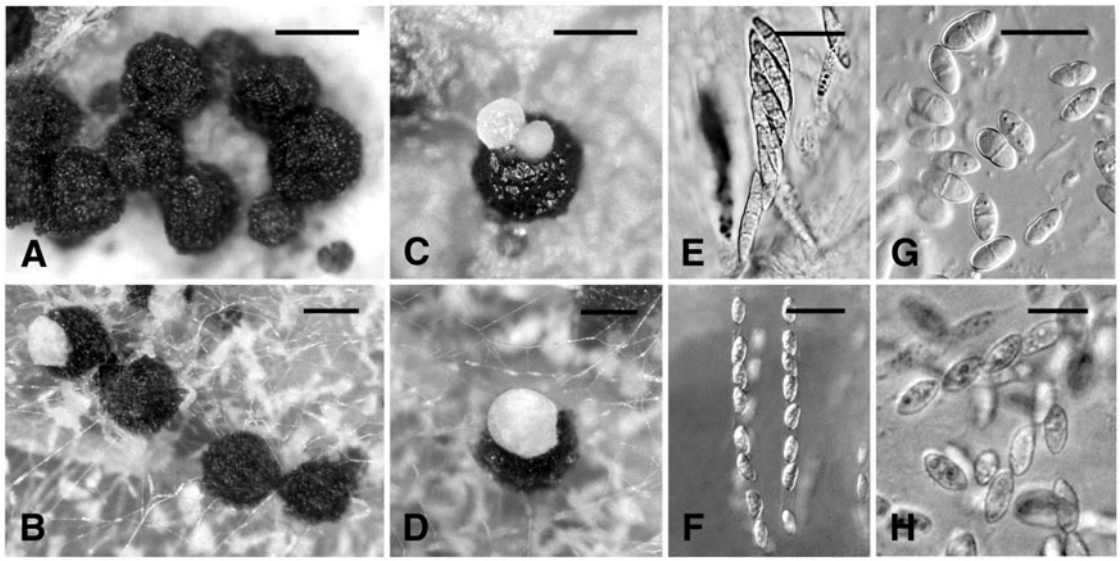

FIGURE 2 Perithecia, asci, and ascospores of *Haematonectria haematococca*, *Gibberella zeae*, and *Gibberella moniliformis*. (A) Cluster of perithecia of *G. zeae* on wheat straw; bar = 200 μm. (B) Perithecia of *H. haematonectria* on carnation leaf pieces from CLA; bar = 200 μm. (C) Perithecium of *G. zeae* oozing ascospores in a cirrhus; bar = 200 μm. (D) Perithecium of *H. haematococca* oozing ascospores in a cirrhus; bar = 200 μm. (E) Asci and ascospores of *G. zeae*, note three-septate ascospores; bar = 25 μm. (F) Asci and ascospores of *H. haematococca*; bar = 25 μm. (G) Ascospores of *G. moniliformis*, note one-septate ascospores; bar = 10 μm. (H) Ascospores of *H. haematococca*; bar = 10 μm. (After Leslie and Summerell, 2006.)

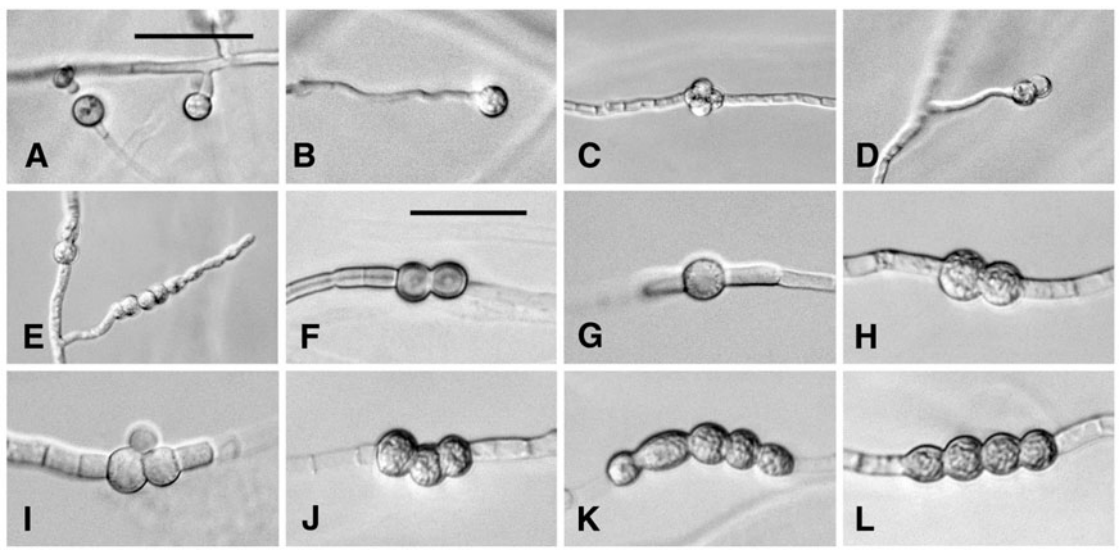

FIGURE 3 Chlamydospores of *Fusarium* species. (A and B) Single, verrucose chlamydospores of *F. solani*; (C and D) clustered chlamydospores of *F. compactum*; (E) chain of verrucose chlamydospores of *F. compactum*; (F) paired, smooth-walled chlamydospores of *F. solani*; (G) single, verrucose chlamydospore of *F. scirpi*; (H) paired, verrucose chlamydospores of *F. compactum*; (I) clustered, smooth-walled chlamydospores of *F. scirpi*; (J and L) chains of verrucose chlamydospores of *F. compactum*; (K) chain of verrucose chlamydospores of *F. scirpi*. Scale bars: panels A through E, 50 μm; panels F through L, 25 μm. (After Leslie and Summerell, 2006.)

TABLE 1 Genome characteristics of four sequenced *Fusarium* species[a]

Fusarium species	Strain	Coverage	Genome size (Mb)	No. of predicted protein-encoding genes	No. of chromosomes	Map available?	% GC
F. graminearum	PH-1[b]	10×	36.45	13,332	4	Yes	48.3
F. oxysporum		6.8×	61.36	17,735	15 ?	No	48.4
N. haematococca MP VI (FGSC9596)	77-13-4	8.2×	52.4	16,237		No	50.8
F. solani			51.29 (Broad)				
F. verticillioides (NRRL 20956)	FGSC7600 (M3125)	8×	41.78	14,179	12	Yes	48.7

[a]Data from http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/MultiHome.html and http://genome.jgi-psf.org/Necha1/Necha1.home.html.
[b]Another strain (Gz3639) was sequenced with a 0.4× coverage for single nucleotide polymorphism analysis.

with these maps playing an important role in the proper assembly of the physical sequences. Chromosome complements within the asexually reproducing species are not as constant as they are in species in which sexual reproduction remains an important part of the life cycle (Boehm et al., 1994).

A number of *Fusarium* spp. also carry chromosomes that are conditionally dispensable and termed variously "CD," "B," or "minichromosomes" (Miao et al., 1991; Xu and Leslie, 1996; Xu et al., 1995). These chromosomes are not known to carry genes for toxin production but can carry genes that are important for the plant pathogenicity process. They also may carry genes responsible for detoxification of plant defense compounds, such as pisatin (Temporini and VanEtten, 2004). CD chromosomes may be irregularly transmitted, lost, or rearranged during meiosis (Miao et al., 1991). There also is evidence that some or all of the genes on such chromosomes may be horizontally transmitted, even across species barriers (Han et al., 2001; Kistler, 2001).

Genetic Manipulations

Most *Fusarium* species are relatively easy to manipulate under laboratory conditions. There are numerous transformation and related protocols available, which vary somewhat from one another, depending upon the marker, the host strain, and the purpose that the transformed strain will serve once it is constructed. Most strains grow well on a mineral-based defined medium (Leslie and Summerell, 2006), although optimal conidiation, production of secondary metabolites, or production of sexual structures or morphological structures for identification purposes often requires more-specialized media.

Sexual crosses usually are made on carrot agar (Klittich and Leslie, 1988), with one strain serving as a female parent and the other as the male. With *F. graminearum*, these protocols are altered somewhat, as a strain with a portion of the mating-type region disabled is used as the female parent to ensure that all of the progeny are the result of a heterozygous cross (Lee et al., 2003). Asci generally contain eight viable spores as an unordered tetrad (Raju, 1994), unless a spore killer gene (Kathariou and Spieth, 1982) or chromosome rearrangement is heterozygous (Bowden et al., 2008). Spores can be collected by shooting from young perithecia for the analysis of unordered tetrads, or as a spore cirrhus from a perithecial ostiole for random spore analyses.

Secondary Metabolites

Fusarium species produce copious quantities of chemically unrelated secondary metabolites. Genes encoding the enzymes that synthesize these secondary metabolites are often found as gene clusters and usually are not distributed across the genome, as is common for genes involved in primary metabolism. Several of these secondary metabolites are important as mycotoxins, whose levels are monitored and regulated in domestic and international commerce (e.g., fumonisins, trichothecenes, and zearalenone), because of their threat to the health of domesticated animals and humans. Two toxins produced by *Fusarium* spp., diacetoxyscirpenol (DAS) and T-2 toxin, are on the United States "select agent" list (www.cdc.gov/od/sap/docs/saist.pdf)). T-2 toxin was associated with the deaths of thousands of Russians consuming contaminated grain after World War II (Gajdusek, 1953; Joffe, 1983), although threats from T-2 and other trichothecenes have been noted since at least the time of ancient Greece (Schoental, 1994).

There are several major classes of secondary metabolites produced by *Fusarium* spp., including polyketides, terpenes, and nonribosomal peptides (Table 2). The effects of these metabolites on plant, animal, and human health have usually been determined on the basis of limited exposure to relatively high levels of individual compounds under tightly defined experimental conditions. However, most *Fusarium* strains are capable of synthesizing multiple secondary metabolites, and the effects of these mixtures may be more important than those of any single compound. For example, beauvericin is limited in its toxicity under most conditions (although yeasts that produce the compound have been proposed for use as insect biocontrol agents) but has properties that enable it to permeabilize cell membranes and thus could increase the potency of some compounds that are not readily taken up by healthy cells (Zhang et al., 2007). Fusaric acid is produced by almost all *Fusarium* species and also may be more important for its ability to permeabilize cells than as a sole pathogenicity factor (Bacon et al., 1996).

In planta activity of zearalenone, and possibly other *Fusarium* secondary metabolites, can be reduced by conjugating the zearalenone with another molecule, often glucose or another sugar (Poppenberger et al., 2006). This conjugation results in the masking of the toxins, as they are no longer detectable as their free forms by routine, validated analytical chemical protocols (Sulyok et al., 2007).

TABLE 2 Some of the best-known secondary metabolites produced by *Fusarium* spp.[a]

Polyketides	Terpenoids	Nonribosomal peptides	Other
Chlamydosporol	Culmorin	Beauvericins	Acuminatum
Equisetins	Cyclonerodiol	Enniatins	Butenolide
Fumonisins	Fusaproliferin		Fusaric acid
Naphthoquinones	Gibberellins		Fusarins
Zearalenone	Trichothecenes (DON, DAS, nivalenol, T-2)		Fusarochromanone
			Moniliformin
			Sambutoxin
			Wortmannin

[a]After Desjardins, 2006.

The fate of these masked compounds in the digestive system of animals has not been intensively studied. These glucosidic linkages may be broken under acidic stomach conditions, freeing the masked mycotoxins to again pose a risk to the animal that consumed them. The formation and stability of these masked mycotoxins thus warrant further evaluation.

Genetic engineering techniques have been used to produce maize plants with increased resistance to stalk and ear rot and with lower levels of fungal colonization (Munkvold, 2003). The first example was that of Bt corn, i.e., maize transformed with a gene from *Bacillus thuringiensis* that conveys resistance to insects. Reducing the insect pressure on the host plant reduces the number of breaches in the outer defense layers of the plant, thus reducing the number of openings by which the fungus can enter the plant. The reduced colonization results in reduced disease pressure and less ear rot, stalk rot, and fumonisin production (Munkvold et al., 1999). A more direct approach to protecting maize plants from accumulating fumonisin is to insert an enzymatic pathway that is capable of breaking down this toxin in the plant as it is synthesized and excreted by the colonizing fungus. Genes encoding such a pathway from *Exophiala spinifera* and *Rhinocladiella atrovirens* have been cloned and transformed successfully into maize, where they can reduce the level of fumonisin contamination (Duvick, 2001). Maize germplasm carrying these exogenous enzymes has not yet been commercialized.

Population Genetics

Populations of *Fusarium* spp. may be genetically diverse or limited with respect to neutral genetic markers, and there are a large number of studies devoted solely to ascertaining the amount of variation present in economically important field isolates. In species in which the sexual stage is known or presumed, including the homothallic *F. graminearum*, most strains differ from others if a sufficiently large number of appropriate polymorphic markers (usually 30 to 50) is scored. Thus, the potential to recombine traits to generate pathogenic characteristics with a multigenic basis is high for most species.

Species with a sexual stage usually have a mixed mode of reproduction whereby both asexual spores and sexual spores can be used for reproduction. Both the relative frequency of the mating-type loci and the frequency of strains that can serve as the female parent in a cross may serve as a constraint to sexual reproduction and lower N_e, the effective population number (Leslie and Klein, 1996). In general, the number of strains capable of functioning as a female parent is a more important constraint than the relative frequency of the mating-type alleles. In species where the ability to cross under laboratory conditions is used as a proxy for crossing under field conditions, the percentage of strains capable of serving as the female parent may be as low as 10%. The proportion of female-fertile strains in the population can be used to estimate the relative frequency of sexual reproduction under field conditions. These low numbers make finding the sexual stage under field conditions quite difficult and the construction of the highly fertile tester strains needed for good descriptions based on the biological species concept an arduous task.

Studies of vegetative compatibility (Leslie, 1993) have been of particular importance in *Fusarium* population analyses. The initial hope was that these markers would be suitable for identifying important strains of all *Fusarium* spp. (Puhalla, 1984, 1985; Puhalla and Spieth, 1983, 1985). Through the use of spontaneously generated mutants that are resistant to chlorate and unable to use nitrate as a sole nitrogen source (*nit* mutants), strains can be checked relatively quickly for their ability to form a heterokaryon and to belong to the same or different vegetative compatibility groups, or VCGs (Correll et al., 1987; Klittich and Leslie, 1988). In populations where sexual recombination occurs, VCGs are useful primarily as markers for strains that have been released and are being tracked in a population, or as a quick way to prove that two strains are not genetically identical. Strains within the same VCG, however, need not be clones (Chulze et al., 2000). VCGs have been most widely applied to populations of *F. oxysporum*, in which members of a single forma specialis often belong to one or a few VCGs. In these instances, the identification of a VCG can be used as a substitute for lengthier and more complicated tests of pathogenicity.

PATHOGENICITY AND VIRULENCE FACTORS

In general, *Fusarium* species are necrotrophic pathogens, i.e., they colonize dead plant (or animal) material. In the past decade, a number of *Fusarium* genes that are important or essential for plant infection have been identified (Roncero et al., 2003). Some of the best-characterized virulence factors in pathogenic strains of *Fusarium* are listed in Table 3.

Genes That Counter Plant Defensive Compounds

Detoxification of phytoalexins is one strategy commonly used by fungal pathogens to overcome plant defense responses. The role of phytoalexin detoxification in pathogenesis has

TABLE 3 Some virulence factors characterized in *Fusarium* species

Gene function	Gene name(s)	Species	Reference(s)
Detoxification	*PDA1*, *MAK1*	*solani*	Temporini and VanEtten, 2004
	TOM1	*oxysporum*	Enkerli et al., 1998
Transporter	*GpABC1*	*sambucinum*	Fleißner et al., 2002
	FcABC1	*culmorum*	Skov et al., 2004
Avirulence gene	*SIX1*	*oxysporum*	Rep et al., 2004
	AVR1 (*SIX4*)	*oxysporum*	Houterman et al., 2008
Hydrolytic enzyme	*FGL1*	*graminearum*	Voigt et al., 2005
MAPK	*GPMK1/MAK1*	*graminearum*	Jenczmionka et al., 2003
	FMK1	*oxysporum*	Di Pietro et al., 2001
	MGV1	*graminearum*	Hou et al., 2002
Trimeric G-protein	*GzGPA2*, *GzGPB1*	*graminearum*	Yu et al., 2008
	GPA1, *GPA2*, *GPB1*	*oxysporum*	Jain et al., 2003, 2005
Monomeric G-protein	*RAS2*	*graminearum*	Bluhm et al., 2007
	RHO1	*oxysporum*	Martinez-Rocha et al., 2008
Trichothecene biosynthesis	*TRI5*	*graminearum*	Proctor et al., 1995
Glucanosyltransferase	*GAS1*	*oxysporum*	Caracuel et al., 2005
Phytotoxic peptide	Esyn1 enniatin synthetase	*avenaceum*	Herrmann et al., 1996
	NEP1	*oxysporum*	
	CPS1	*graminearum*	Lu et al., 2001
Chitinase	*CHS2*, *CHS7*, *CHSV*, *CHSVb*	*oxysporum*	Martin-Udiroz et al., 2004; Martin-Urdiroz et al., 2008
Protein kinase	*SNF1*	*oxysporum*	Ospina-Giraldo et al., 2003
Amino acid and nucleotide synthesis	*CBL1*, *RSY1*, *GzHIS7*, *ARG2*, *ADE5*	*graminearum*	
pH regulation	*PacC*	*oxysporum*	Caracuel et al., 2003b
Iron metabolism	*NPS6*, *NPS2*	*graminearum*	Oide et al., 2006;
	SID1		Greenshields et al., 2007
Protein degradation	*FBP1*	*graminearum*	Han et al., 2007
	FRP1	*oxysporum*	Duyvesteijn et al., 2005
Miscellaneous factors	*FOW1*	*oxysporum*	Inoue et al., 2002
	FOW2	*oxysporum*	Imazaki et al., 2007
	FPD1	*oxysporum*	Kawabe et al., 2004
	FSR1	*graminearum*, *verticillioides*	Shim et al., 2006
	CLC1	*oxysporum*	Canero and Roncero, 2008

been most extensively studied in *F. solani* (*Nectria haematococca* mating population VI) isolates pathogenic on pea. Highly virulent strains can detoxify the pea phytoalexin pisatin. Disruption of the *PDA1* gene, which encodes a phytochrome 450 monooxygenase with pisatin demethylation ability, reduces the virulence (Wasmann and VanEtten, 1996). Additional *PDA* genes (*PDA2* to *PDA7*) involved in pisatin detoxification also have been identified. All of these genes contribute to increased virulence towards pea and are located on a conditionally dispensable chromosome. In *F. solani* isolates virulent to chickpea, the detoxification of maackiain also contributes to virulence (Enkerli et al., 1998). Four genes (*MAK1* to *MAK4*) encoding proteins capable of detoxifying maackiain have been identified. The *MAK1* gene, which encodes a flavin-containing monooxygenase, also is located on a conditionally dispensable chromosome. The conditionally dispensable chromosome of *F. solani* appears to contain many pea pathogenicity genes that are important for host range determinants but are dispensable for normal growth in culture (Temporini and VanEtten, 2004).

In the tomato pathogen *F. oxysporum*, detoxification of the phytoalexin tomatine also is an important virulence factor. The *F. oxysporum* genome encodes five putative tomatinase genes, identified as *TOM1-5* (Pareja-Jaime et al., 2008). Mutants lacking *TOM1* have reduced tomatinase activities and virulence on tomato, with delays in symptom development, and have increased sensitivity to tomatine (Pareja-Jaime et al., 2008).

An alternative to detoxifying plant defensive compounds is to preferentially export them out of the cell. In *Gibberella pulicaris*, the *GpABC1* gene encodes an ATP-binding cassette (ABC) transporter that is required for virulence on potato tubers (Fleißner et al., 2002). Mutants disrupted in the *GpABC1* gene are sensitive to the phytoalexin rishitin and infect potatoes poorly, although they still can metabolize rishitin. The *FcABC1* gene, a homologue of *GpABC1* in *F. culmorum*, also is an important virulence factor (Skov et al., 2004). Deletion of *FcABC1* has no effect on fungal growth but significantly reduces virulence on flowering wheat heads.

Signal Transduction Pathways

Several well-conserved signal transduction pathways have been studied in some *Fusarium* species. Mitogen-activated protein kinase (MAPK) genes homologous to the *M. grisea PMK1* have been characterized in *F. oxysporum* (*FMK1*) and *F. graminearum* (*GPMK1/MAK1*). The *FMK1* gene is dispensable for conidiation, conidium germination, and vegetative growth by *F. oxysporum* but is required for the differentiation of penetration hyphae and root attachment (Di Pietro et al., 2001). *fmk1* mutants are not pathogenic towards tomato and have significantly reduced expression of the pectate lyase gene *PL1*. Deletion of *FMK1* has no obvious effect on virulence to immunodepressed mice (Prados-Rosales et al., 2006), which indicates that this MAPK pathway does not have the same role in animal infection as it does in plant infection. In *F. graminearum*, *GPMK1/MAK1* regulates mating, conidiation, and pathogenesis (Jenczmionka et al., 2003; Urban et al., 2003). Δ*gpmk1/mak1* mutants conidiate poorly, have reduced ability to colonize flowering wheat heads, are female sterile, and cannot infect roots, wounded wheat floral tissues, or tomato fruits. One of the genes regulated by Gpmk1 is *FGL1*, which encodes a secreted lipase required for full virulence of *F. graminearum* (Voigt et al., 2005).

Two other MAPK genes (*MGV1* and *OS2*) have been characterized in *F. graminearum*, but only *MGV1* is required for plant infection (Hou et al., 2002). The *mgv1* mutant accumulates less of the trichothecene deoxynivalenol (DON), is hypersensitive to the plant defensin MsDef1, and is defective in hyphal fusion and heterokaryon formation. Mutants blocked in the *FgOS2* MAPK pathway are capable of infecting plants normally but produce lower amounts of trichothecene mycotoxins on rice medium (Ochiai et al., 2007). These mutants have enhanced pigmentation in aerial hyphae and elevated expression of genes involved in the biosynthesis of the pigment aurofusarin.

The cyclic AMP-protein kinase A (cAMP-PKA) pathway also regulates fungal development and pathogenesis. In *F. solani*, pharmacological studies indicate that cAMP signaling may be important in responses to flavonoids exuded from legume roots, including spore germination (Bagga and Straney, 2000). Treatment with flavonoids transiently increases the intracellular cAMP levels in macroconidia, probably due to inhibition of cAMP phosphodiesterase. However, molecular studies are needed to confirm these observations. There are no published studies of the genes encoding adenylate cyclase or the catalytic or regulatory subunits of PKA in *Fusarium*. In *F. graminearum*, preliminary analyses indicate that both adenylate cyclase and the catalytic subunit of PKA are important for plant infection (Z. Zhou and J.-R. Xu, unpublished data).

Heterotrimeric G-proteins normally function upstream from the cAMP-PKA pathway and/or MAPK cascades. In *F. graminearum*, the Gα gene *GzGPA2* and Gβ gene *GzGPB1* are required for full virulence (Yu et al., 2008). The other two Gα genes, *GzGPA1* and *GzGPA2*, are dispensable for plant infection. *GzGPB1* and *GzGPA1* also reduce female fertility and mycotoxin biosynthesis. Δ*GzGPB1* deletion mutants have reduced vegetative growth but produce higher levels of DON and zearalenone. In *F. verticillioides*, loss of the *GBB1* Gβ gene leads to reduced fumonisin production but does not alter virulence (Sagaram and Shim, 2007). In *F. oxysporum*, the Gβ gene *FGB1* plays a regulatory role in various developmental and plant infection processes (Jain et al., 2003). The Δ*fgb1* mutant has reduced virulence and conidiation but increased resistance to heat. It also has defects in hyphal growth and colony morphology. Similar phenotypes were observed in the *fga1* Gα mutant (Jain et al., 2002), but only the *fgb1* mutant is aconidial. Unlike the *fgb1* or *fga1* mutants that retain reduced virulence, pathogenicity was completely lost in the *fga2* Gα mutant, which has normal colony morphology and conidiation (Jain et al., 2005). The *fgb1* mutant has normal Fmk1 phosphorylation levels, and exogenous cAMP partially restores its growth phenotype (Delgado-Jarana et al., 2005), indicating that Fgb1 acts upstream of the cAMP-PKA pathway, but not the Fmk1 Map kinase.

Two monomeric G proteins also have been implicated in *Fusarium*-plant interactions. In *F. graminearum*, *RAS2* plays an important role in conidium germination, vegetative growth, and plant infection (Bluhm et al., 2007). *RAS2* is involved in activation of the Gpmk1 MAPK and the expression of the *FGL1* lipase gene. In *F. oxysporum*, the Rho1 GTPase is an important virulence factor on tomato plants but is dispensable for the infection of immunosuppressed mice (Martinez-Rocha et al., 2008). The Δ*rho1* mutant has a reduced growth rate and cell wall defects that can be partially relieved by the osmotic stabilizer sorbitol.

Cell Wall- and Cutin-Degrading Enzymes

For phytopathogenic fungi, breaching the plant surface and cell wall often is the first step in plant infection. All of the sequenced *Fusarium* genomes contain multiple copies of genes encoding cutinases, xylanases, polygalacturonases (PG), and other cell wall-degrading enzyme (CWDE) genes, indicating the importance of these hydrolytic enzymes. However, it is difficult to determine the role of any individual cutinase or other CWDE in plant pathogenesis due to their redundancy. Disruption of one gene may increase the expression of other genes with overlapping functions and have little or no effect on overall enzymatic activities during plant infection. In the vascular wilt pathogen *F. oxysporum*, none of the endopolygalacturonase and exopolygalacturonase genes that have been functionally characterized are essential for virulence on tomato plants (Garcia-Maceira et al., 2001). Similarly, all of the xylanase genes studied by targeted disruption in *F. oxysporum* are dispensable for plant infection (Gomez-Gomez et al., 2002). In *Aspergillus nidulans*, XlnR is the major transcriptional activator for the expression of xylanase genes. Deletion of the XlnR orthologue in *F. oxysporum* alters xylanase gene expression but has no detectable effect on virulence (Calero-Nieto et al., 2007).

Although disruption of a cutinase gene in *F. solani* f. sp. *pisi* was reported to reduce its virulence on pea (Rogers et al., 1994), results regarding the role of cutinase in pathogenesis by this fungus are inconsistent (Stahl et al., 1994). In *F. oxysporum*, disruption of the yeast *SNF1* homologue significantly reduces virulence towards cabbage and *Arabidopsis* (Ospina-Giraldo et al., 2003). As has been reported for *Cochliobolus carbonum*, *SNF1* regulates CWDE gene expression. In yeast, the Snf1 protein kinase is required for the transcription of many glucose-repressed genes (e.g., for thermotolerance and peroxisome biogenesis), and the inability of *snf1* mutants of *F. oxysporum* to infect plants may be due to many factors other than changes in CWDE activities.

Cell Wall Synthesis/Integrity

The fungal cell wall is the first barrier against plant defense responses, so maintaining cell wall integrity is important for fungal pathogens. In *F. oxysporum*, four of the six characterized chitin synthase genes (*CHS1*, *CHS2*, *CHS3*, *CHS7*, *CHSV*, and *CHSVb*) are required for full virulence. The *chs2*, *chs7*, *chsV*, and *chsVb* mutants grow normally in vitro

but have reduced virulence on tomato plants. Some of these mutants are defective in stress responses, resistance to plant defense compounds, or other developmental and plant infection processes (Madrid et al., 2003; Martin-Udiroz et al., 2004; Martin-Urdiroz et al., 2008). The *chsV* disruptant can rapidly kill both immunocompetent and immunosuppressed mice (Martin-Urdiroz et al., 2008), suggesting enhanced animal virulence in the mutant (and perhaps an importance for this chitin synthase in either vegetative growth or plant pathogenicity). The *GAS1* gene, which encodes a putative β-1,3-glucanosyltransferase, is required for plant infection by *F. oxysporum* (Caracuel et al., 2005). The Δ*gas1* deletion mutant has restricted colony growth and elevated transcript levels of *CHSV* and *RHO1*, which help maintain cell wall integrity. The cell wall defect associated with Δ*gas1* mutants is alleviated by the osmotic stabilizer sorbitol.

Phytotoxic Metabolites and Peptides

Some *Fusarium* mycotoxins also are toxic to plant cells, although molecular genetic studies of their role in pathogenicity are generally limited to the trichothecene and fumonisin toxins.

To date, there are two secondary metabolites that are clearly associated with plant diseases: DON, with fusarium head scab of wheat and barley, and gibberellic acid, with bakanae disease of rice. Fusarium head scab has been linked to DON in two ways. First there were mechanistic studies by Proctor et al. (1995, 2002) showing that strains that produced more DON were more aggressive than strains that produced nivalenol as an alternative to DON or that produced no trichothecenes whatsoever. Second, in a quantitative trait locus analysis, Cumagun et al. (2004) showed that the cluster of genes responsible for trichothecene biosynthesis was at the heart of the only major quantitative trait locus in a cross between fungal strains that differed in their ability to cause fusarium head scab.

Gene disruption experiments indicate that production of DON is an important virulence factor in *F. graminearum*. Mutants carrying a deletion of the trichodiene synthase gene *TRI5* are blocked in DON synthesis and impaired in wheat and maize infection (Harris et al., 1999; Proctor et al., 1995). Although production of DON is not necessary for initial infection, it is important for the spread of the fungus within colonized wheat spikes (Bai et al., 2002). The *tri5* mutant remains virulent on barley (Maier et al., 2006) and on maize grown under hot, dry environmental conditions (Proctor et al., 2002), suggesting that DON has both a host- and an environment-dependent role during plant infection.

Natural defenses against trichothecene toxins include acetylation at position C-3 by trichothecene 3-*O*-acetyltransferase, which is encoded by the *Tri101* gene or its presumed homologues in a number of plant and fungal systems (Garvey et al., 2008). The protection offered by these homologues against trichothecenes is usually not complete, with the effects of the trichothecene toxin usually being reduced rather than completely eliminated.

Bakanae disease results from the overproduction of gibberellic acid, a plant growth promoter, by *Fusarium fujikuroi* when it colonizes rice seedlings (Graebe, 1987; Spector and Phinney, 1968). The excess of plant growth promoter results in elongated stems that lodge easily and whose grain usually fails to fill. A hot-water treatment of rice seed prior to planting reduces or eliminates the endogenous *Fusarium* inoculum during early plant growth and suffices to prevent the disease.

Fumonisins can cause apoptosis in plant cells (Wang et al., 1996). However, the contribution of fumonisins to the virulence of *F. verticillioides* on corn is complex. The fumonisin-deficient mutant is as virulent as the wild type on maize in seedling blight assays, and insensitivity to fumonisin B_1 (FB_1) in maize has no direct impact on resistance to *F. verticillioides* infection (Desjardins et al., 2007; Proctor et al., 2002). Nevertheless, FB_1 producers colonize seedlings more effectively than do fumonisin-nonproducing strains. *F. verticillioides* strains from banana lack a portion of the fumonisin biosynthetic gene cluster, i.e., from *FUM19* to *FUM21*, and cannot cause foliar disease symptoms on maize seedlings. Reintroducing the entire fumonisin gene cluster into a banana isolate results in transformants that produce fumonisins and that are pathogenic on maize seedlings (Glenn et al., 2008), suggesting that fumonisins may play a role in foliar infection of maize. *F. graminearum* lacks the entire fumonisin gene cluster but retains portions of the flanking *ZBD1* and *ORF21* sequences (Waalwijk et al., 2004). It is likely that the *FUM* cluster was lost through independent events in these two fungi.

Fusarium species also produce toxic peptides and proteins. Enniatins are phytotoxic cyclohexadepsipeptides produced by some *Fusarium* species. In *F. avenaceum*, the enniatin synthetase gene is required for full virulence on potato tubers (Herrmann et al., 1996). However, the role of enniatin in pathogenesis of other enniatin-producing fungi is not clear. The *CPS1* gene encodes a peptide synthetase-like protein that is well conserved in a number of fungal pathogens. Although its exact biochemical function is not clear, *CPS1* is required for full virulence by *F. graminearum* and other fungi (Lu et al., 2001). The product synthesized by *CPS1* must play a role in fungus-plant interactions.

Nep1 is an extracellular protein produced by *F. oxysporum* that induces necrosis and ethylene production in coca, tobacco, and other dicot plants (Bailey, 1995; Jennings et al., 2001). Dose-dependent necrosis is observed in tobacco leaves infiltrated with Nep1 proteins. Induction of active oxygen species and cell death are observed in tobacco cell cultures treated with high concentrations of Nep1. Nep1-like proteins (NLPs) are predicted to belong to the cupin superfamily and have been identified in a variety of plant pathogens, including fungi, bacteria, and oomycetes (Cechin et al., 2008). Members of the NLP family contain a common NPP domain and can induce hypersensitive-like responses in various dicot plants. In *Arabidopsis*, Nep1 inhibits both root and cotyledon growth and triggers cell death. Treatment with Nep1 alters the expression of a number of genes, including some of those involved in reactive oxygen species production, ethylene biosynthesis, and apoptosis (Bae et al., 2006). However, the exact three-dimensional structure and function of NLPs remain to be determined.

General Metabolism and Nutrient Uptake

In *F. graminearum*, several genes involved in primary metabolism, such as *CBL1*, *RSY1*, *GzMETE*, *GzHIS7*, *ADE5*, and *ARG2*, which are required for methionine, adenine, and arginine biosynthesis, also are important virulence factors (Kim et al., 2007; Seo et al., 2007; Seong et al., 2005). An *F. oxysporum* arginine auxotrophic mutant also is reduced in virulence (Namiki et al., 2001). These observations indicate that the uptake of amino acids or nucleotides from plant tissue by *Fusarium* pathogens is limited during disease development. The high-mobility-group-coenzyme A reductase gene *HMR1* is an essential gene in *F. graminearum* (Seong et al., 2006). Disruption of the N-terminal portion of the *HMR1* gene severely reduces growth rate and virulence. Thus, the changes in virulence

observed for the *hmr1* mutant could be directly related to its growth defects.

NPS6 encodes a nonribosomal peptide synthetase that synthesizes the extracellular siderophores of *F. graminearum* (Oide et al., 2006). The Δ*nps6* mutant is hypersensitive to H_2O_2 and is less virulent than the wild type. Homologues of *NPS6* from other fungal pathogens also play an important role in plant infection (Oide et al., 2006). Exogenous iron increases the virulence of the *nps6* mutant on wheat. Therefore, extracellular siderophores synthesized by *NPS6* participate in iron uptake or sequestration of iron from the host plant but do not act directly as phytotoxins. In *F. graminearum* and other *Fusarium* species, another NPS gene, *NPS2*, is responsible for the synthesis of ferricrocin (Tobiasen et al., 2007). *NPS2* probably plays a minor role in plant pathogenesis, because the *nps2* mutant is slightly less virulent than is the wild-type strain from which it was derived. Two genes with roles in iron metabolism, *SID1* and *FET3*, also have been characterized in *F. graminearum* (Greenshields et al., 2007), but only *SID1* is required for full virulence.

pH Regulation

Homologues of the PacC/Rim1 zinc finger protein are well conserved in fungi as the major transcriptional regulator of the pH response and have been functionally characterized in both *F. oxysporum* (Caracuel et al., 2003b) and *F. verticillioides* (Flaherty et al., 2003). In both fungi, the Δ*pac*C mutants grow poorly at alkaline pH. Expression of a dominant active allele of *Pac*C in *F. oxysporum* results in an alkalinity-mimicking phenotype with reduced virulence. The Δ*pac*C mutant has an acidity-mimicking phenotype and is more virulent than its wild-type parent in root infection assays (Caracuel et al., 2003b). The *pac*C mutant also is more sensitive to Li^+ and Na^+ and fails to activate transcription of the *ENA1* gene or maintain ion homeostasis in *F. oxysporum* at a high pH (Caracuel et al., 2003a). In *F. verticillioides*, the *pac1* mutant was not tested for virulence but produces more fumonisin than the wild-type parent when grown on maize kernels and in a synthetic medium buffered at pH 4.5. The mutant also can produce FB_1 when cultured at pH 8.4, a condition inhibitory to fumonisin synthesis in the wild type (Flaherty et al., 2003). These observations suggest that PacC homologues may negatively regulate plant infection and secondary metabolism in at least some *Fusarium* species.

Protein Degradation

In both *F. oxysporum* (Frp1) and *F. graminearum* (Fbp1), an F-box protein similar to *Saccharomyces cerevisiae* Grr1 is required for pathogenesis (Duyvesteijn et al., 2005; Han et al., 2007). The *frp1* mutant is defective in root colonization but exhibits normal growth and conidiation (Duyvesteijn et al., 2005). *FBP1* is required by *F. graminearum* for sexual development, plant infection, and other processes. In yeast, Grr1 is a component of the Skp1-Cullin-F-box protein (SCFGrr1) E3 ligase complex that is necessary for protein degradation. Fbp1 and Frp1 interact with yeast Skp1 or its homologue in *F. graminearum*, suggesting that the SCFGrr1 complex may be conserved in these fungi for the ubiquitin-mediated degradation of proteins involved in pathogenesis or virulence. However, phenotypes of the *frp1* and *fbp1* mutants are not identical, suggesting that this well-conserved complex may regulate distinct processes in different fungi.

Avirulence Genes

While many *Fusarium* species have no race substructure, some have well-defined races and avirulence genes. In *F. oxysporum* f. sp. *lycopersici*, at least two avirulence genes have been identified. *SIX1* (secreted in xylem 1) encodes a small, cysteine-rich protein secreted by *F. oxysporum* during the colonization of xylem vessels. SIX1 is the avirulence gene corresponding to the *I-3* resistance gene in tomato (Rep et al., 2004). A second avirulence gene, *AVR1*, encodes a secreted protein that triggers resistance responses in tomato plants carrying the *I* or *I-1* resistance gene (Houterman et al., 2008). The Avr1 protein also is a virulence factor that functions as a suppressor of disease resistance mediated by the *I-2* and *I-3* R genes. *SIX2* and *SIX3* also encode proteins secreted in the xylem by *F. oxysporum* f. sp. *lycopersici*, but their functions are not yet known. *SIX1* and *SIX2* are in a small gene cluster (7.8 kb) together with the *SHH1* (salicylate hydroxylase homologue 1) gene, while *SIX3* is located elsewhere on the same chromosome. All of these genes are unique to *F. oxysporum* f. sp. *lycopersici* strains that are pathogenic on tomato (van der Does et al., 2008).

Miscellaneous Virulence Factors

Some virulence factors identified in *Fusarium* species are novel genes or have no clear biochemical function. For example, *FPD1* encodes a putative transmembrane protein in *F. oxysporum* (Kawabe et al., 2004) and *FSR1* encodes a protein with seven WD40 repeats in *F. verticillioides* and *F. graminearum* (Shim et al., 2006). In *F. oxysporum*, the *FOW1* and *FOW2* genes were identified by random insertional mutagenesis to be required for plant infection (Imazaki et al., 2007; Inoue et al., 2002). *FOW1* encodes a mitochondrial protein that is highly conserved in filamentous fungi. Fow2 is a nuclear protein with the Zn_2Cys_6 motif. Although these genes are important for pathogenesis, their exact roles in fungal plant infections remain to be determined. Similarly, the mode of action for the *CLC1* gene in *F. oxysporum*, which encodes a putative CLC-type voltage-gated chloride channel (Canero and Roncero, 2008) is unknown, although *clc1* mutants cannot infect tomatoes and are hypersensitive to oxidative stress.

CONCLUDING REMARKS

Fusarium spp. are a diverse group of fungi. To date, four *Fusarium* species have been sequenced, which is more than for any other genus of plant pathogenic fungi. The sequencing of an additional two *F. oxysporum* isolates has recently been funded. Some of these fungi have well-developed classical genetic systems and genetic maps that will allow relatively rapid correlations to be made between physical sequences and fungal phenotypes. The existence of many characterized populations of these fungi means that natural variants, as well as laboratory-induced mutants, should be available to provide complementary views of fungal pathogenesis and the genes responsible. The presence of economically important strains in natural settings also offers the possibility of understanding the transition of these fungi from pathogens in mixed stands of grasses and other plants to the efficient killing machines found in many monoculture agriculture settings.

Considering the importance of many *Fusarium* diseases and the diverse fungus-plant interactions in which they participate, it is reasonable to expect that additional *Fusarium* species and strains will be sequenced in the near future. As the sequences of more and more genomes become available, comparative genomics approaches can be used to study the genetic basis of various developmental and plant infection processes, including production of different types of spores

(chlamydospores, macroconidia, and microconidia), race and host specificity, and endophytic growth. As various *Fusarium* species cause different types of diseases that rely on different mechanisms and host interactions, comparative genomic studies may be essential to identify and understand the molecular mechanisms underlying *Fusarium*-plant interactions.

A simple comparison of the genome sizes of four sequenced *Fusarium* species shows that *F. oxysporum* and *F. solani* have larger genomes than do *F. graminearum* and *F. verticillioides* (Table 1). Dispensable or B chromosomes were first described in *F. solani*. A number of pea infection-related genes (PEP genes) are located on a single dispensable chromosome. Some of these sequences may have been obtained from, or donated to, other organisms by horizontal gene transfer. As a second example, in *F. oxysporum* the *SIX3* gene and the 7.8-kb region encoding *SIX1*, *SIX2*, and *SHH1* are located on the same chromosome. The *SIX1* region is flanked by repetitive elements (Rep et al., 2004). These sequences are unique to *F. oxysporum* isolates that are pathogenic on tomato and may have originated from a horizontal gene transfer event (van der Does et al., 2008). The *F. oxysporum* genome contains a higher percentage of repetitive sequences and many more active transposable elements than does any of the other sequenced *Fusarium* genomes. In contrast, the *F. graminearum* genome has no active transposable elements and the lowest percentage of repetitive sequences (0.1%) of all sequenced filamentous fungi. Genome rearrangement and duplication are presumably rare in *F. graminearum*, which may explain why its genome has organized regions of genes with different recombination frequencies and potential roles in plant infection (Cuomo et al., 2007).

Genetics- and genomics-based approaches will certainly further improve our understanding of molecular mechanisms and evolution of *Fusarium* pathogenesis. Results from these basic studies will provide information critical to the design of new disease control methods. For example, knowledge gained from the elucidation of the trichothecene mycotoxin biosynthesis pathway resulted in the transformation of wheat with the *TRI101* trichothecene 3-*O*-acetyltransferase genes (Okubara et al., 2002). Transgenic plants expressing *TRI101* have improved disease resistance. Because mycotoxin contamination is a serious concern with crops infected by some *Fusarium* species, approaches that do not control disease development but eliminate or reduce mycotoxin production may have important practical applications.

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Ustilago maydis and Maize: a Delightful Interaction

FLORA BANUETT

Ustilago maydis (DeCandole) Corda is a basidiomycete fungus belonging to the Ustilaginomycotina, the smut fungi. This group consists of plant pathogenic fungi that attack more than 75 families of flowering plants, both dicots and monocots. Smut diseases of cereal grains (wheat, barley, sorghum, oats, maize, and rice) and sugar cane are the best known of the smut diseases because of their economic impact (Agrios, 2005). The smut fungi derive their name from the smutty appearance of the dark spores produced at the end of their infectious cycle (see below). These spores are diploid and are the major source of inoculum; they can be spread by wind and rain and are resistant to adverse environmental conditions, a property conferred by their specialized cell wall (Christensen, 1963). Another common characteristic of the smut fungi is their ability to undergo a dimorphic switch, a feature they share with fungal pathogens of humans, such as the ascomycetes *Candida albicans* and *Histoplasma capsulatum* and the basidiomycete *Cryptococcus neoformans*.

The Ustilaginomycotina (smuts; described above) and the Pucciniales (rusts) are two major groups of plant pathogen basidiomycete fungi. The rusts are characterized by their complex life cycle, including the production of various spore and cell types, their obligately parasitic mode of growth, and, in some cases, the requirement for two hosts to complete the life cycle. The smuts and rusts share a biotrophic mode of growth; that is, they grow in the host plant without killing it (Agrios, 2005). Other biotrophic fungi include the mycorrhyzal fungi (beneficial fungi), many of which are members of the Basidiomycota, and the powdery mildews (pathogenic ascomycete fungi). The smut fungi have a narrow host range; for example, *U. maydis* (maize, teosinte), *U. avenae* (oats), *U. nuda* (barley), *U. tritici* (wheat), *U. hordei* (barley), and *U. scitaminea* (sugar cane); *Sporisorium sorghi* (sorghum), and *Sporisorium reilianum* (maize and sorghum). What properties of these different hosts influence the ability of a particular smut fungus to invade and induce symptom development? What functions do different smut fungi possess that restrict their interaction to a narrow set of hosts?

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U. maydis differs from most other well-characterized smut fungi in two major aspects: (i) it can produce teliospores (diploid spores) in vegetative plant parts, whereas other smut fungi require the inflorescence for teliospore production; and (ii) it induces tumor formation, whereas other smut fungi do not (Fig. 1). In nature, tumor formation in monocots is extremely rare; it is more common, though still rare, in dicotyledonous plants. The ability of *U. maydis* to alter growth control of the host has elicited great interest. What genetic information allows *U. maydis* to alter growth control of the host plant? Is it production of plant growth regulators or transfer of DNA? Likewise we can ask, what conditions make the tumor tissue a propitious environment for teliospore formation?

U. maydis is the etiological agent of corn smut disease or cuitlacoche (reviewed by Banuett, 2002). There are only two known hosts of the fungus: maize (*Zea mays* L.) and teosinte (*Zea mays* subsp. *parviglumis* and subsp. *mexicana*), the progenitor of maize (Banuett, 2002; Christensen, 1963). The disease is characterized by tumors that occur on all aerial plant parts (Fig. 1), and the infected cobs have been a culinary delicacy in Mexico since ancient times (see Banuett, 1992; and Ruiz-Herrera and Martínez-Espinoza, 1998). *U. maydis*, as well as other smuts, has been the subject of intensive research since the 18th century (see Banuett, 2007, and references therein). It was extensively studied at University Farm, University of Minnesota, from the 1920s to 1950s by pioneers such as J. J. Christensen, E. C. Stakmann, W. F. Hanna, J. B. Rowell, and J. E. DeVay. The last two demonstrated the genetic basis of mating-type incompatibility. Studies of *U. maydis* were also carried out in Germany by O. Brefeld, H. O. Sleumer, and R. Bauch. Later studies by P. Day, J. Puhalla, S. Anagnostakis, and D. Perkins in the United States and by R. Holliday and colleagues in the United Kingdom rekindled interest in *U. maydis* (reviewed by Banuett, 2007). The development of the first *Escherichia coli-U. maydis* shuttle vector, procedures for uptake of exogenous DNA, and demonstration of one-step gene replacement by S. Leong and colleagues at the University of Wisconsin ushered in the era of molecular genetics in *U. maydis* (reviewed by Banuett, 2002). These procedures paved the way for cloning and molecular analysis of the mating-type loci, the master regulators of the

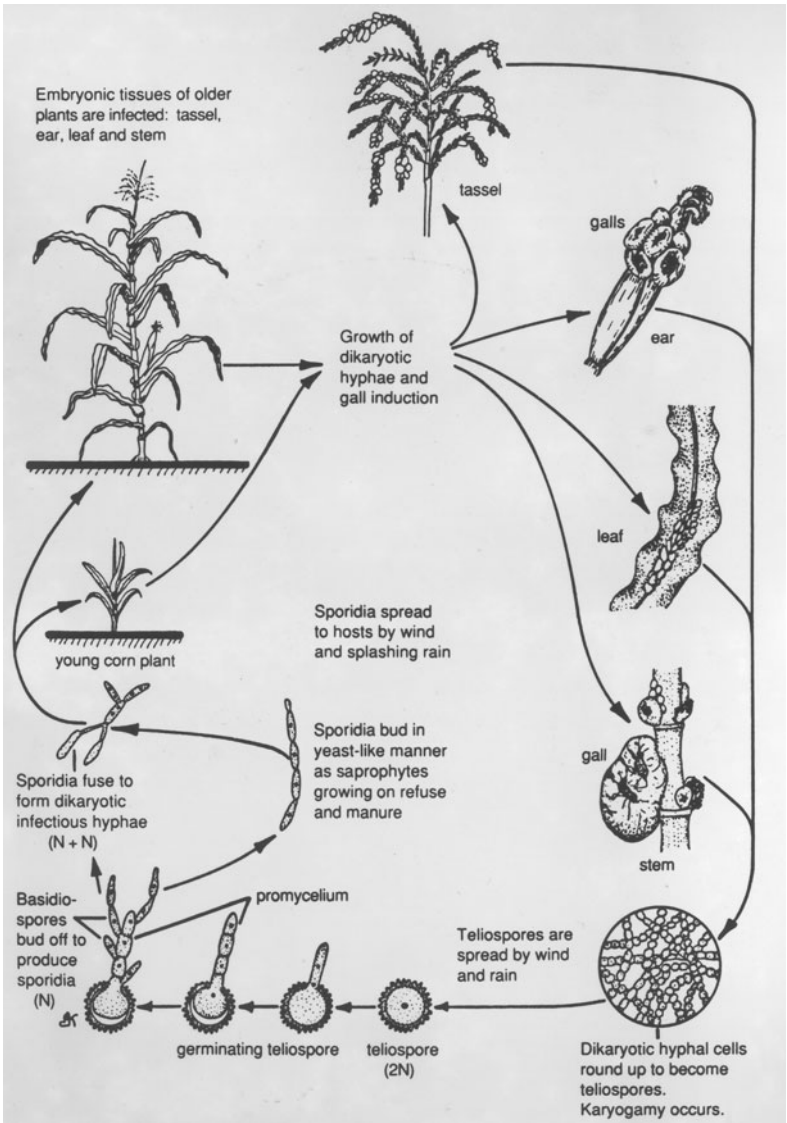


FIGURE 1 Life cycle of *U. maydis*. Modified with permission from Kenaga et al., 1971.

life cycle, by S. Leong, J. W. Kronstad, R. Kahmann, I. Herskowitz, and F. Banuett (reviewed by Banuett, 2007). Since then, there has been a veritable explosion of information on various aspects of the *U. maydis* life cycle. Despite these advances, little is known of the molecular mechanisms by which the fungus alters growth control of its host.

Completion of the life cycle of *U. maydis* requires passage through the plant; therefore, pathogenicity and the life cycle are intimately intertwined (Fig. 1 and 2). *U. maydis* can exist as a yeast-like unicellular form and as a filamentous form. The switch from one form to the other entails a change in ploidy (haploid versus dikaryon), ability to induce disease (nonpathogenic versus pathogenic), and growth habit (saprophytic and free-living versus biotrophic and parasitic). Thus, dimorphism, nuclear state, pathogenicity, and growth habit are all interrelated aspects of the life cycle (Fig. 2). Understanding the molecular mechanisms of the interaction of *U. maydis* with maize entails understanding the molecular mechanisms that regulate its life cycle (Fig. 1 and 2).

This chapter presents an overview of our current knowledge regarding the interaction of *U. maydis* with maize. Due to space limitations, only selected aspects of the published work are discussed. First, useful features that have facilitated analysis of the life cycle are described, followed by a brief synopsis of the morphological transitions that characterize the life cycle and their control by the mating-type loci and the mitogen-activated protein kinase (MAPK) and cyclic AMP (cAMP) signal transduction pathways. Lastly, specific genes known to be required or to be expressed at different stages of the infectious cycle are described.

USEFUL FEATURES

Several features of *U. maydis*, combined with development of tools for its molecular genetic manipulation, have made it an attractive organism for the study of the interaction of pathogenic biotrophic fungi with their hosts (reviewed by Banuett, 2002). *U. maydis* is genetically tractable owing to the existence of a haploid unicellular form that has a short

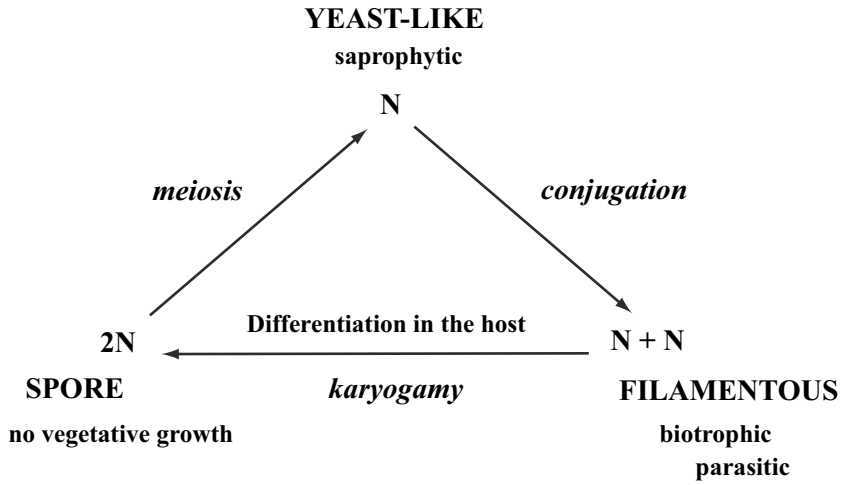


FIGURE 2 Life cycle transitions in *U. maydis*. Three basic forms characterize the life cycle of *U. maydis*: a yeast-like cell, a filamentous form, and a spore (teliospore). The transition from one form to the other is accompanied by changes in ploidy, growth habit, and ability to induce tumors and entails three processes: meiosis, conjugation, and karyogamy, respectively. The fungus undergoes additional morphological changes in the host that are not observed in culture, suggesting that host signals play an important role in fungal differentiation.

generation time (120 min in rich medium), comparable to that of the best-studied eukaryotes: *Saccharomyces cerevisiae* (90 min) and *Schizosaccharomyces pombe* (120 min). *U. maydis* haploids form compact colonies on different solid media, allowing the application of standard microbiological techniques. DNA-mediated transformation with shuttle vectors and different dominant selectable markers (*hyg*, hygromycin; *ble*, phleomycin; *cbx*, carboxin; and *nat*, nourseothricin) and the ability to replace wild-type genes with mutant versions by homologous recombination permit the generation of multiple knockout or specific mutations in one strain. The availability of two regulatable promoters, *Pnar1* and *Pcrg1*, allows the generation of conditional mutations, a particularly valuable tool for the study of essential genes. Stable diploids can be constructed in the laboratory and used for complementation studies and to analyze the role of genes in postfusion events (see below). Under controlled environmental conditions, the life cycle can be completed in 2 to 3 weeks, facilitating studies of the infectious process in the vegetative parts of the plant (reviewed by Banuett, 2002). Availability of the genome sequence (Kämper et al., 2006) has accelerated discovery of gene function by using a candidate gene approach in combination with global genome approaches to study cellular processes such as cell cycle, cell polarity and the cytoskeleton, cell wall synthesis, DNA recombination and repair, evolution of mating types, meiosis, metabolism, the posttranscriptional machinery, secondary metabolism, the secretome, signal transduction, and telomere organization and function (see *Fungal Genetics and Biology*, volume 45, supplemental issue on *U. maydis*, for articles on these topics; for cell cycle, see Pérez-Martín et al., 2006).

LIFE CYCLE AND THE INFECTIOUS PATHWAY

Overview of the Morphological Transitions

Three major cell types characterize the life cycle of *U. maydis*: a haploid unicellular yeast-like form that divides by budding and is nonpathogenic; a dikaryotic filamentous form that divides at the apical cell and is pathogenic; and a round diploid cell, the teliospore, which is not capable of vegetative growth but undergoes meiosis to produce the haploid cell (Fig. 2). Generation of these forms involves meiosis, conjugation, and karyogamy, respectively, and is accompanied by changes in ploidy, growth habit, pathogenicity, and ability to undergo meiosis (Fig. 2). Understanding the life cycle of *U. maydis* entails understanding the molecular mechanisms that regulate these intimately intertwined processes. As described below, the repertoire of morphologies is increased by interaction with the plant (Fig. 3A), which has led to the hypothesis that plant signals regulate morphology and differentiation of the fungus. Furthermore, the fungus also alters morphology of the host (Banuett and Herskowitz, 1996; Ruiz-Herrera et al., 1999).

The salient features of the infectious cycle are described in the following section. Genes known to be involved in this process are discussed later, in "The Infectious Process: Dramatis Personae."

The Infectious Process: a Drama in Five Acts

The infectious process of *U. maydis* can be divided into five major steps: (i) fusion of haploid cells to generate the filamentous dikaryon and proliferation on the plant surface; (ii) formation of appressorium-like structures and invasion of host cells; (iii) formation of clamp-like structures, intracellular proliferation, and tumor induction; (iv) production of mucilaginous material, karyogamy, hyphal fragmentation, cell rounding, deposition of a specialized cell wall, and production of mature teliospores; (v) teliospore germination, meiosis, and generation of the haploid form (Fig. 3).

Two unlinked mating-type loci, *a* and *b*, regulate diverse aspects of the life cycle. In order to complete the life cycle, haploid strains that fuse to generate the infectious filamentous dikaryon must carry different alleles at both *a* and *b*, for example *a1 b1* + *a2 b2* (see "The Mating-Type Loci: Master Regulators of Life Cycle Transitions" below) (reviewed by Banuett, 2002; Kahmann and Kämper, 2004; and

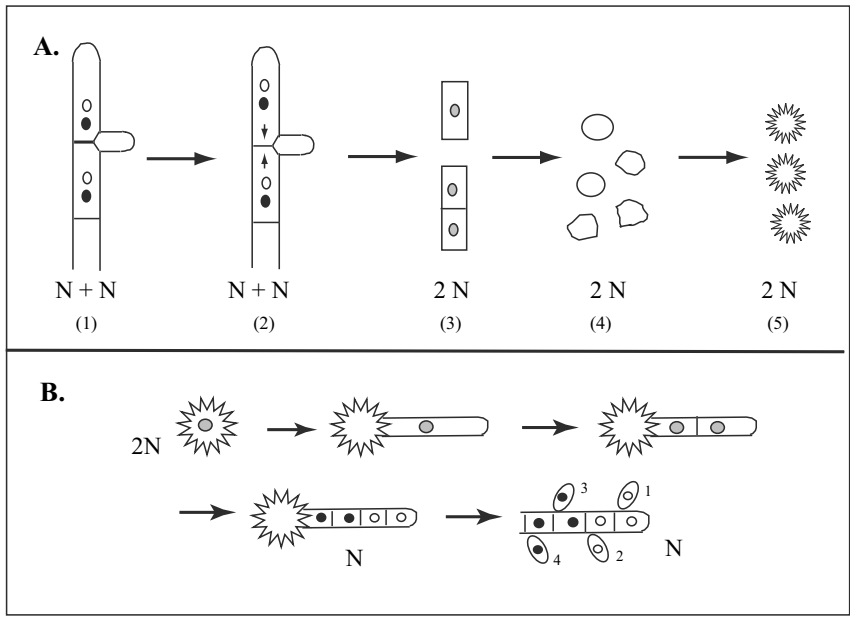


FIGURE 3 Fungal differentiation in the plant. (A) Fungal hyphae develop appressorium-like structures prior to penetration (not shown; see the text for details). Clamp-like structures (1 and 2) (short branches with Y-shaped septum) are necessary for nuclear partitioning and proliferation (see the text for details). The fungus branches profusely prior to tumor induction. Once tumors are formed, fungal hyphae undergo fragmentation, releasing cylindrical fragments containing a single nucleus (3). These fragments then undergo cell rounding (4) and deposit a specialized cell wall resulting in formation of mature diploid teliospores (5). Arrows point to likely sites of cell wall-remodeling events prior to fragmentation. (B) The teliospore germinates by formation of a short filament, the promycelium. The diploid nucleus migrates into the promycelium to complete meiosis resulting in a four-cell septate promycelium. These four haploid cells are the primary meiotic products. They give rise, by budding, to basidiospores, which in turn produce chains of yeast-like cells by budding. The progeny cells can be isolated by micromanipulation and used for segregation analysis.

Klosterman et al., 2007). The input of the mating-type loci in early steps of the life cycle (cell fusion and formation of the filamentous dikaryon) has been studied outside the plant, exploiting the ability of the haploid form to grow in a variety of microbiological media and the existence of stable diploids that mimic the behavior of the dikaryon. These studies have shown that cell fusion is governed by the *a* locus and that filamentous growth is controlled by both *a* and *b* in culture, but only by *b* in the plant (see "The Mating-Type Loci" below) (reviewed by Banuett, 1995, 2002; Kahmann and Kämper, 2004; and Klosterman et al., 2007). It should be noted that the morphology of the filamentous dikaryon or diploid in culture differs from that in the plant.

Cell Fusion of Haploids and Generation of the Infectious Filamentous Dikaryon

In the laboratory, 5- to 10-day-old maize seedlings are inoculated with a mixture of haploid strains that carry different *a* and *b* alleles using a hypodermic syringe. The cells develop conjugation tubes similar to those formed in culture (see "The *a* Locus" below) (Banuett and Herskowitz, 1994a, 1994b; Snetselaar, 1993). Cell fusion is mediated by conjugation tubes and is observed as early as 8 h postinoculation; dikaryotic hyphae arise from the clumps of mating yeast-like cells and proliferate on the leaf surface. The hyphae branch soon after they emerge from the yeast-like clusters, where cell fusion has occurred (Banuett and Herskowitz, 1996). This observation contrasts with that of others that indicate that branching does not occur on the leaf surface (reviewed by Kahmann and Kämper, 2004). The reason for this difference is not known, but it may be due to different maize lines used in these studies (see below). Hyphal branching does not occur in culture, suggesting that the leaf surface or a plant hormone induces this morphological differentiation (Banuett and Herskowitz, 1996). Infections can be carried out with solopathogenic strains (strains that induce tumors in pure culture) such as diploids heterozygous at *a* and *b*, or haploids engineered to contain different *a* and *b* alleles (reviewed by Banuett, 2002). Recent work has shown that lipids induce filamentous growth in culture (Klose et al., 2004). Interestingly, diploids heterozygous at both mating-type loci in the presence of lipids form hyphae that exhibit morphological features observed exclusively during fungal growth in the plant (see "Clamp-Like Structures, Fungal Proliferation within Host Cells, and Tumor Induction" below).

The first detectable symptom of infection is chlorosis, a yellowing of the green tissue, that can be observed as early as 24 h after inoculation (Banuett and Herskowitz, 1996; Christensen, 1963). Production of a toxin was reported to be responsible for chlorosis (see Banuett, 2002, for reference). The fungus also induces the formation of anthocyanin pigmentation in maize lines of the appropriate genetic background. The pigment may be part of a defense response against fungal invasion. An increase in pectate lyase was reported to accompany chlorosis and teliospore production, and higher polygalacturonase levels accompany anthocyanin production,

tumor formation, and teliospore production (Cano-Canchola et al., 2000). Recent mRNA profiling analysis of infected tissue has shown that genes coding for different cell wall-degrading enzymes are upregulated to different levels during tumor formation (Doehlemann et al., 2008b). Interestingly, an endopolygalacturonase showed the highest level of induction during growth in the plant. Deletion of the gene coding for this enzyme had no effect on pathogenicity. Double and triple mutants lacking genes coding for endopolygalacturonase, pectin lyase, and methyl esterase had no effect on pathogenicity. These enzymes thus appear to play little or no role in pathogenicity, or partially redundant functions are able to substitute for them.

The filamentous dikaryon grows on the leaf surface until penetration. Some studies indicate that under these conditions, the dikaryon consists of a straight-growing, unbranched filament with a long apical cell that contains cytoplasm and two nuclei and subapical compartments devoid of cytoplasm (reviewed by Kahmann and Kämper, 2004; and Pérez-Martín et al., 2006). Because this mode of growth resembles that in culture, where the nuclei are arrested in the G_2 phase of the cell cycle (García-Muse et al., 2003a), it was concluded that the nuclei of the filament in planta are also cell cycle arrested (reviewed by Kahmann and Kämper, 2004). In contrast, other studies show that the newly formed filaments exhibit a different morphology from those in culture: they can branch and contain compartments with cytoplasm prior to penetration of host cells (Banuett and Herskowitz, 1996), suggesting that release from the putative cell cycle arrest can occur before penetration. Because different maize lines were used in these studies, these observations point to a major role of the host in modulation of fungal growth and differentiation. Microscopic examination of the infectious cycle in different maize cultivars may lead to a better understanding of host input.

Formation of Appressorium-Like Structures and Penetration of Host Cells

Penetration occurs directly through or between plant cells (reviewed by Banuett, 2002) and involves the development of appressorium-like structures, a swelling of the hyphal tip (Brachmann et al., 2003; Snetselaar and Mims, 1994). Penetration can also occur through stomata and wounds (Banuett and Herskowitz, 1996, and references therein); whether appressorium-like structures are involved during invasion of cells via these venues remains to be determined. The *U. maydis* appressoria do not resemble the highly differentiated structures formed by other pathogenic fungi, such as the rusts and the ascomycete *Magnaporthe grisea*. The appressoria in *M. grisea* are capable of exerting great mechanical force for penetration. It is not known if the appressorium-like structures in *U. maydis* produce lytic enzymes to aid in penetration.

Clamp-Like Structures, Fungal Proliferation within Host Cells, and Tumor Induction

After penetration of the host cell, the hyphal tips come in intimate contact with the host membrane (Kämper et al., 2006; Schirawski et al., 2005; Snetselaar and Mims, 1994). Indeed, in other smut fungi, a zone of interaction has been described (Bauer et al., 1997), and it appears to be present in the *U. maydis*-maize interaction (Kämper et al., 2006; Schirawski et al., 2005). This region is likely the site for the exchange of signals between fungus and plant cell and also the site for acquisition of nutrients by the fungus. *U. maydis* does not form haustoria, which are highly differentiated hyphae, developed by the rusts, that participate in nutrient acquisition and exchange of other molecules (see Kemen et al., 2005).

As described above, some studies indicate that the infectious hypha consists of a long tip cell with two nuclei followed by subapical compartments devoid of cytoplasm and that the nuclei are arrested in the G_2 phase of the cell cycle. Release from this arrest is proposed to ensue after penetration, allowing nuclear division to take place, resulting in formation of a hypha with dikaryotic compartments (reviewed by Kahmann and Kämper, 2004). Regardless of whether nuclear division occurs before or only after penetration, nuclear distribution requires the development of clamp-like structures (Scherer et al., 2006). In *U. maydis*, the clamp-like structure is a short branch that forms at the site of the future septum that will separate the apical from the subapical cell (Fig. 3A). After division of the two genetically distinct nuclei in the tip cell, one pair remains in the future apical cell. Of the remaining pair, one nucleus migrates subapically to the future subapical cell, and the partner nucleus migrates into the clamp-like structure, which at this point lacks a septum. A Y-shaped septum develops and separates these three cells (Fig. 3A). The nucleus in the clamp-like structure migrates to the subapical cell, perhaps through a septal pore or after induced septal degradation, and dikaryosis is reestablished in the subapical cell (Scherer et al., 2006).

Clamp-like structures in *U. maydis* had been previously described (Banuett and Herskowitz, 1996; Snetselaar and Mims, 1994) but were not thought to be involved in nuclear partitioning because of failure to fuse with the subapical cell, as occurs in the basidiomycetes *Schizophyllum commune* and *Coprinus cinereus*. The studies with *U. maydis* demonstrate that fusion of the clamp-like cell is not a requisite for its involvement in nuclear partitioning (Scherer et al., 2006). Formation of clamp-like structures is independent of the presence of different *a* alleles (Banuett and Herskowitz, 1996; Scherer et al., 2006), consistent with observations that different *a* alleles are not needed for the infectious process. Formation of clamp-like structures in *U. maydis* requires growth in the plant: it does not occur in culture, which suggests that plant inputs are involved in this morphogenetic differentiation (Banuett and Herkowitz, 1996; Scherer et al., 2006). The signal may be lipids: diploids heterozygous at *a* and *b* respond to lipids in culture by formation of septate, branched hyphae, which form clamp-like structures (Klose et al., 2004). This is the first demonstration that a specific compound induces a hyphal morphology normally restricted to the plant and offers the possibility of studying the genetic control of this morphogenetic differentiation. It remains to be determined if the clamp-like structures formed in vitro participate in nuclear portioning.

The fungus can proliferate intra- and intercellularly and exhibits extensive branching during this stage (Banuett and Herskowitz, 1996; Doehlemann et al., 2008b; Snetselaar and Mims, 1994). Proliferation of the fungus leads eventually to tumor induction, which can be detected as early as 3 to 5 days after inoculation (Banuett and Herskowitz, 1996; Doehlemann et al., 2008b). The trigger that leads to alteration of growth control of the host is not known. Mutations in several genes block or attenuate tumor induction, but the amino acid sequence of their gene products has failed to provide any clues about the molecular mechanisms involved (see below). The predicted small, secreted proteins coded for by clusters of genes uncovered during analysis of the genome sequence could act as effectors of tumor induction

(see below) (Kämper et al., 2006). The tumors induced by *U. maydis* are veritable neoplastic transformations that involve increased cell divisions and cell enlargement (see Banuett and Herskowitz, 1996, and references therein), which has led to the hypothesis that the fungus produces plant growth regulators or modifiers of endogenous plant growth regulators that interfere with normal cell division and growth (reviewed by Banuett, 1995). The availability of the *U. maydis* genome sequence provides the opportunity to explore these possibilities. It remains to be determined whether these neoplastic transformations are capable of hormone-independent and fungus-independent growth in culture. Tumors arise on different plant parts, including the leaf sheath, leaf blade, stems, and floral parts. Infection of floral parts can induce sexual transformation, where tassels produce kernels and cobs produce tassels. In addition, infection of anthers at various developmental stages not only induces tumors but can also cause aberrant development of different parts of the anther (V. Walbot, personal communication). Thus, infections with *U. maydis* may provide important insights about floral development in maize. The hypothesis has been put forth that perhaps some of the above-mentioned clusters of genes code for signals that mimic endogenous plant signals that modulate development via interaction with leucine-rich repeat (LRR) receptors of unknown function and interfere with normal development (V. Walbot, personal communication).

Teliospore Production: a Carefully Orchestrated Developmental Pathway

In large tumors, the hyphae become embedded in a mucilaginous material, have a convoluted appearance, and may exhibit lobbed tips. The hyphae fragment at this stage, releasing cylindrical fragments of one to four cells (Fig. 3A). Karyogamy takes place most likely prior to hyphal fragmentation, as suggested by the fact that each cylindrical cell contains a single nucleus. The presence of a single nucleus is indicative of the start of the diploid stage of the fungus. The cylindrical fragments undergo cell rounding (Fig. 3A). The rounded cells deposit a specialized echinulated cell wall (Fig. 3A), which first is yellowish brown and later becomes dark brown (Banuett and Herskowitz, 1996; Snetselaar and Mims, 1994). This cell wall has been shown to be different from the cell wall in yeast-like cells and hyphae (reviewed by Banuett, 1995). It provides protection against adverse environmental conditions, allowing the teliospore to survive in the soil for many years (Christensen, 1963).

Hyphal fragmentation occurs within the tumors (Banuett and Herskowitz, 1996), though it has been reported that this process can occur between cells (Doehlemann et al., 2008b; Snetselaar and Mims, 1994). Because different maize lines were used in these studies, it suggests that the host can modulate the course of infection. The mucilaginous material may serve as osmotic stabilizer during the cell wall-remodeling events that occur during teliospore maturation (Banuett and Herskowitz, 1996). The origin of this mucilage is not known. *fuz1* mutants do not produce it (see below), which suggests a fungal origin, but other scenarios are also possible (Banuett and Herskowitz, 1996). The morphogenetic changes leading to formation of teliospores likely involve rearrangements of the secretory machinery: first, from the hyphal tip during proliferation, to the septal region during fragmentation, and then to the entire cell surface during cell rounding and isotropic deposition of the cell wall. These hypothesized rearrangements in the localization of the secretory machinery most likely reflect changes in the organization of the cytoskeleton and cell polarity factors.

Teliospore Germination, Meiosis, and Generation of the Haploid Phase

The teliospore is the natural diploid phase in the life cycle of *U. maydis* (Fig. 2 and 3). It is approximately 7 to 8 μm in diameter and has a thick cell wall consisting of three layers (reviewed by Banuett, 1995). The teliospore germinates by extrusion of the promycelium or metabasidum, a short filament of 20 to 25 μm in length and 2 to 3 μm in diameter (Fig. 3B). The cell wall is presumably dissolved at the point of emergence of the promycelium. The nucleus, which is arrested in late prophase of meiosis I, migrates from the body of the teliospore to the promycelium, where it completes the meiotic divisions (Fig. 3B). During these divisions, there is partial breakdown of the nuclear envelope, as occurs during budding in basidiomycete yeasts (reviewed by Banuett, 1995). A septum with a narrow pore forms at the end of each division, resulting in a four-septate structure at the end of meiosis. These are the primary meiotic products (Fig. 3B). Progeny cells (basidiospores) arise from each of these primary meiotic products by budding, producing chains of haploid yeast-like cells (Fig. 3B), which can be isolated by micromanipulation for tetrad analysis. During nuclear division, the nucleus migrates from the promycelial cell to the basidiospore, where it divides, with subsequent migration of one of the nuclei to the promycelial cell (reviewed by Banuett, 1995). The pattern of teliospore germination varies among different isolates.

Teliospores can germinate directly on the leaf surface and start the infectious process after cell fusion of progeny of opposite mating type. Teliospores can also germinate and undergo meiosis on laboratory media, which greatly facilitates segregation analysis (Banuett and Herskowitz, 1989; reviewed by Christensen, 1963). Competence to undergo meiosis is acquired by passage through the plant, but meiosis itself can take place outside the plant. The diploid state and heterozygosity at the mating-type loci are not sufficient for meiosis: diploids constructed in the laboratory cannot undergo meiosis unless they infect a plant and induce tumors (Banuett and Herskowitz, 1989, 1996).

Little is known about the signals that trigger germination and release from meiotic prophase I arrest. One possibility is that karyogamy, which occurs in the plant, triggers initiation of meiosis, but subsequently, a plant signal induces arrest of meiosis until the proper signals for germination and release from meiotic arrest are perceived. Nutrients, osmoticum, or the leaf surface may relieve the block and allow completion of meiosis. The presence of sugars and other carbon sources appears to stimulate germination (see Zahiri et al., 2005, and references therein). Recent expressed sequence tag analysis has identified a large number of genes that are expressed during teliospore germination (see "Differential Gene Expression during Teliospore Germination" below) (Zahiri et al., 2005).

Pathogenicity of Dikaryons versus Diploids: the Host Has the Last Word

In culture, the dikaryotic filament is short-lived, but diploids heterozygous at *a* and *b* form filaments that are more stable than the dikaryotic filaments on charcoal agar (see below). The reason for this difference is not known; both dikaryons and diploids contain the same genetic information, though in the dikaryon it is compartmentalized in two distinct nuclei, whereas in the diploid it is contained within a single

nucleus. Several reports have suggested that these two filamentous forms differ in pathogenicity, with the dikaryon being more pathogenic than the diploid; however, the strains used in such studies were not isogenic. Two studies using the same congenic strains have not resolved this issue. In one study (Banuett and Herskowitz, 1996), no differences in symptom development were detected when a time course of infection of dikaryons and diploids was compared. In the other investigation (Babu et al., 2005), the diploid exhibited delayed symptom appearance and, overall, a less vigorous response. One important difference between these studies is that different maize lines were used. Babu et al. (2005) conclude: "there may be differences in how the diploids and dikayrons detect and respond to variation in host genotypes." This is likely to be the case. Other observations indicate that different maize lines influence the response to fungal infection (F. Banuett, unpublished observations). Indeed, researchers at University Farm, University of Minnesota, in the 1940s had documented different responses of maize lines to *U. maydis* (reviewed by Banuett, 2002). Babu et al. (2005) detected differences in gene expression between the diploid and dikaryotic filamentous forms in culture; these differences may perhaps relate to differences in symptom development on different maize cultivars by dikaryons and diploids.

THE MATING-TYPE LOCI: MASTER REGULATORS OF LIFE CYCLE TRANSITIONS

Two unlinked mating-type loci, *a* and *b*, regulate diverse aspects of the life cycle. In order to complete the life cycle, haploid strains that fuse must carry different alleles at both *a* and *b*, for example, *a1 b1* + *a2 b2* (reviewed by Banuett, 2002, 2007). The input of the mating-type loci is modulated by nutrition, pH, temperature, oxygen tension, and plant signals (reviewed by Banuett, 2002; Kahmann and Kämper, 2004; and Klosterman et al., 2007).

The *b* Locus

The *b* locus has 25 naturally occurring alleles. Each allele contains two genes, *bW* and *bE*, that code for distinct homeodomain polypeptides; for example, the *b1* allele contains *bW1* and *bE1*, and the *b2* allele contains *bW2* and *bE2*. The bW and bE polypeptides from the same allele cannot interact; only those contributed by different alleles interact to form a heterodimeric homeodomain protein, from here on referred to as "the active b protein" or "b protein." Thus, in a *b1/b2* dikaryon there are two heterodimers, bW1-bE2 and bW2-bE1, either one of which is sufficient to activate *b*-dependent expression (reviewed by Banuett, 2002, 2007; Kahmann and Schirawski, 2007; and Klostermann et al., 2007). Because the *b* locus is the major determinant of filamentous growth in culture and in planta, and also of pathogenicity, it was proposed that *b* regulates expression of genes that more directly control these processes (reviewed by Banuett, 1995, 2002; and Kahmann and Schirawski, 2007). Indeed the *b* locus controls expression of several genes, which fall into two classes: class 1 genes are directly regulated by b by virtue of containing a b binding site (*bbs*), and class 2 genes are indirectly regulated by b, lack a *bbs* site, and are proposed to be regulated by regulatory proteins encoded by class 1 genes (Brachmann et al., 2001). One major regulator of class 2 genes is Rbf1, a zinc finger protein (J. Kämper, personal communication).

Class 1 genes identified thus far include the following (the potential or known function of the predicted protein is indicated in parentheses): *dik6* (no similarities to proteins in the databases), *polX* (a putative DNA polymerase), *lga2* (no similarities to proteins in the databases), and *rbf1*. Class 2 genes include *egl1* (an endoglucanase), *dik1* (no similarities to known proteins), *rep1* (repellent protein), *hum1* (a hydrophobin), *pdi1* (hypothetical disulfide isomerase), *kpp6* (a MAPK required for penetration), *exc1* (hypothetical exochitinase), *frb63* (unknown function), *frb172* (hypothetical K^+/H^+ antiporter), *pma1* (hypothetical plasma membrane ATPase), *atr1* (hypothetical acyltransferase), *frb110* (unknown function), *cap1* (unknown function), *frb124* (unknown function), *mfa1* and *mfa2* (a1 and a2 pheromone precursors, respectively), and *pra1* and *pra2* (a1 and a2 pheromone receptors, respectively) (Brachmann et al., 2001, and references therein). Eleven of these genes have been deleted, and only *kpp6* and *rbf1* are required for pathogenicity (see "The Infectious Process: Dramatis Personae" below) (Brachmann et al., 2003; reviewed by Kahmann and Kämper, 2004; J. Kämper, personal communication). It is likely that additional regulators remain to be uncovered and that an interplay of Rbf1 and other regulators controls the pathogenic program.

The *b* locus regulates the first step in the infectious process; filamentous growth of the pathogenic dikaryon (see "Formation of the Infectious Dikaryon" below). Little is known of the role of the *b* locus at different stages of the infectious cycle, except that it appears to be required for normal meiosis (Day et al., 1971). Given that competence for meiosis is acquired by passage through the plant, the studies of Day et al. (1971) suggest a role of *b* in acquisition of competence to undergo meiosis. One approach to understand the role of *b* during various stages of the infectious cycle may be to control its expression with stage-specific promoters. The *b* locus remains a major and fascinating puzzle in studies of the life cycle of *U. maydis*.

The *a* Locus

The *a* locus has two alleles, *a1* and *a2*, and each allele codes for components of a pheromone response pathway (a pheromone precursor and a pheromone receptor gene) (Bölker et al., 1992; reviewed by Banuett, 2002). Different *a* alleles are required for conjugation tube formation and for cell fusion (Banuett and Herskowitz, 1994a, 1994b; Spellig et al., 1994; Snetselaar, 1993; Snetselaar et al., 1996). Pheromones induce a morphological response, i.e., formation of conjugation tubes. The nucleus locates at the base of the conjugation tube (Banuett and Herskowitz, 1994a, 1994b; Snetselaar, 1993) and is arrested in the G_2 phase of the cell cycle (García-Muse et al., 2003a). The position of the nucleus in cells responding to pheromones contrasts with that in actively budding cells, in which it locates to the cell middle for most of the cell cycle. Conjugation tube formation is solely dependent on the presence of different *a* alleles. Upon cell fusion mediated by the conjugation tubes, the nuclei migrate towards the emerging cell to establish the dikaryon; migration of the nuclei appears to be dependent on the *a* locus (reviewed by Banuett, 2002). The fate of the resulting dikaryon depends on the *b* locus: if different *b* alleles are present, straight, fast-growing dikaryotic hyphae with a long tip cell containing cytoplasm and small subapical compartments devoid of cytoplasm are formed; if identical *b* alleles are present, distorted, slow-growing, multinucleate hyphae form, and these degenerate quickly (Banuett and Herskowitz, 1994b; Snetselaar, 1993). The *a* locus also governs maintenance of filamentous growth on charcoal agar (Banuett and Herskowitz, 1989; Bölker et al., 1992; Spellig et al., 1994). Given

that the dikaryon contains two different pheromone precursor and two different pheromone receptor genes, this requirement indicates that maintenance of filamentous growth is controlled by an autocrine-like response (Bölker et al., 1992; Spellig et al., 1994). Interestingly, different *a* alleles are not necessary for filamentous growth in the plant (see also "Formation of the Infectious Dikaryon" below) (Banuett and Herskowitz, 1996; Regenfelder et al., 1997).

The Charcoal Agar Assay: an Assay for Filamentous Growth

Formation of dikaryotic filaments is a two-step process that requires cell fusion and filamentous growth per se. Mutations that alter the first step also affect the second step, but mutations that alter filamentous growth do not necessarily affect cell fusion (Banuett and Herskowitz, 1989, 1994a, 1994b; reviewed by Banuett, 1995, 2002). To assay the first step, charcoal broth or water agar can be used (Banuett and Herskowitz, 1994a, 1994b; Snetselaar, 1993); to assay filamentous growth, charcoal agar is used. On this medium, costreaking or cospotting of strains carrying different *a* and *b* alleles (for example, *a1 b1* + *a2 b2*) results in a fuzzy reaction ("the fuzz reaction") due to the formation of dikaryotic filaments (a Fuz^+ phenotype), whereas strains carrying different *a* alleles and identical *b* alleles (for example, *a1 b1* + *a2 b1*) or identical *a* alleles and different *b* alleles (for example, *a2 b1* + *a2 b2*) do not form filaments (a Fuz^- phenotype) (Fig. 4) (Banuett and Herskowitz, 1989). Because strains that carry different *a* alleles and identical *b* alleles are able to fuse but do not produce a fuzz reaction, this plate assay is not a true measure of cell fusion. To determine if a gene is required for filamentous growth, independently of any requirement for cell fusion, diploid strains heterozygous at *a* and *b* (for example, *a1/a2 b1/b2*) or haploids engineered to carry different alleles at both mating-type loci (for example, *a1 mfa2 bW1 bE2*) that bypass the need for cell fusion are commonly used (Banuett and Herskowitz, 1989, 1994a, 1994b; Regenfelder et al., 1997; Dürrenberger et al., 1998). The dikaryotic filaments formed on charcoal agar are short-lived; diploids heterozygous at both loci are more long-lived than the dikaryons but eventually give rise to yeast-like cells. These observations support the notion that plant signals are crucial for maintenance of filamentous growth of the dikaryon or diploid.

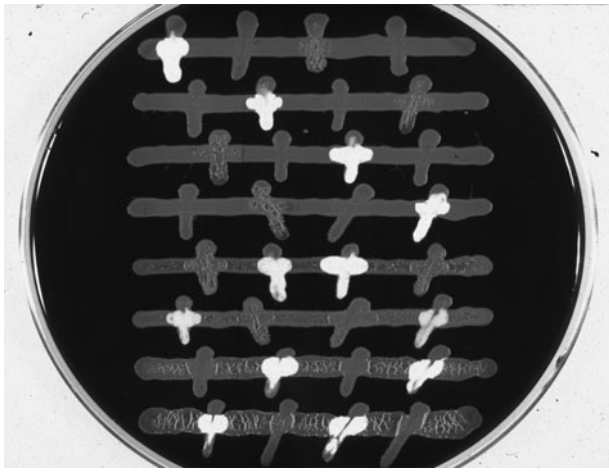


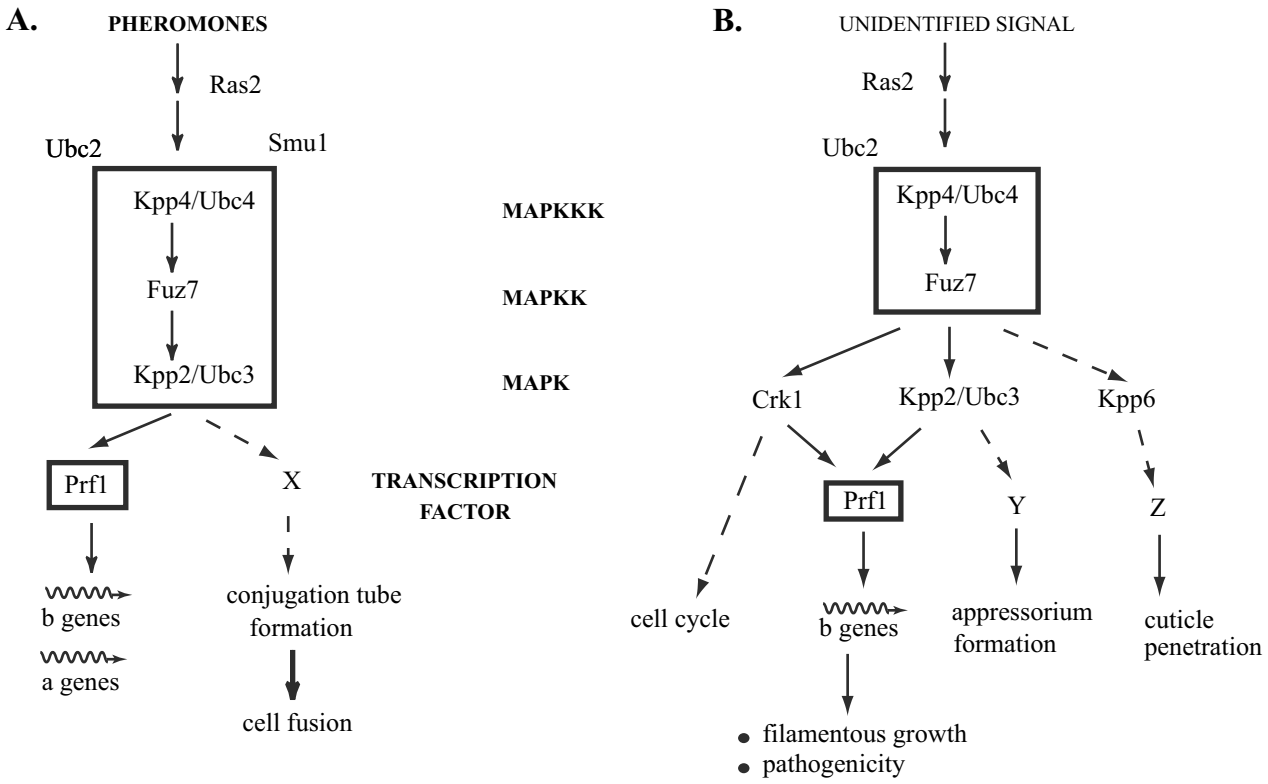
FIGURE 4 Formation of filaments on charcoal agar. Saturated cultures of haploid strains (top four horizontal lines) and Fuz^- diploids (bottom four horizontal lines) were costreaked against haploid testers *a1 b1*, *a2 b2*, *a1 b2*, and *a2 b1* on charcoal medium and incubated overnight at room temperature. Strains in the horizontal lines are (from top to bottom) *a2 b2*, *a1 b1*, *a2 b1*, *a1 b2*, *a1/a2 b1/b1*, *a1/a2 b2/b2*, *a1/a1 b1/b2*, and *a2/a2 b1/b2*. The fuzzy reaction observed is due to formation of filaments. Haploid strains that carry different *a* and *b* alleles form dikaryotic filaments when costreaked on this medium (top four reactions). Diploid strains heterozygous at *a* and homozygous at *b* or homozygous at *a* and heterozygous at *b* form filaments when costreaked with haploid strains that carry a different *b* allele (regardless of the *a* allele) or a different *a* allele (regardless of the *b* allele), respectively (bottom four reactions). Diploids heterozygous at both *a* and *b* form mycelial colonies (not shown). Reproduced from Banuett and Herskowitz, 1989.

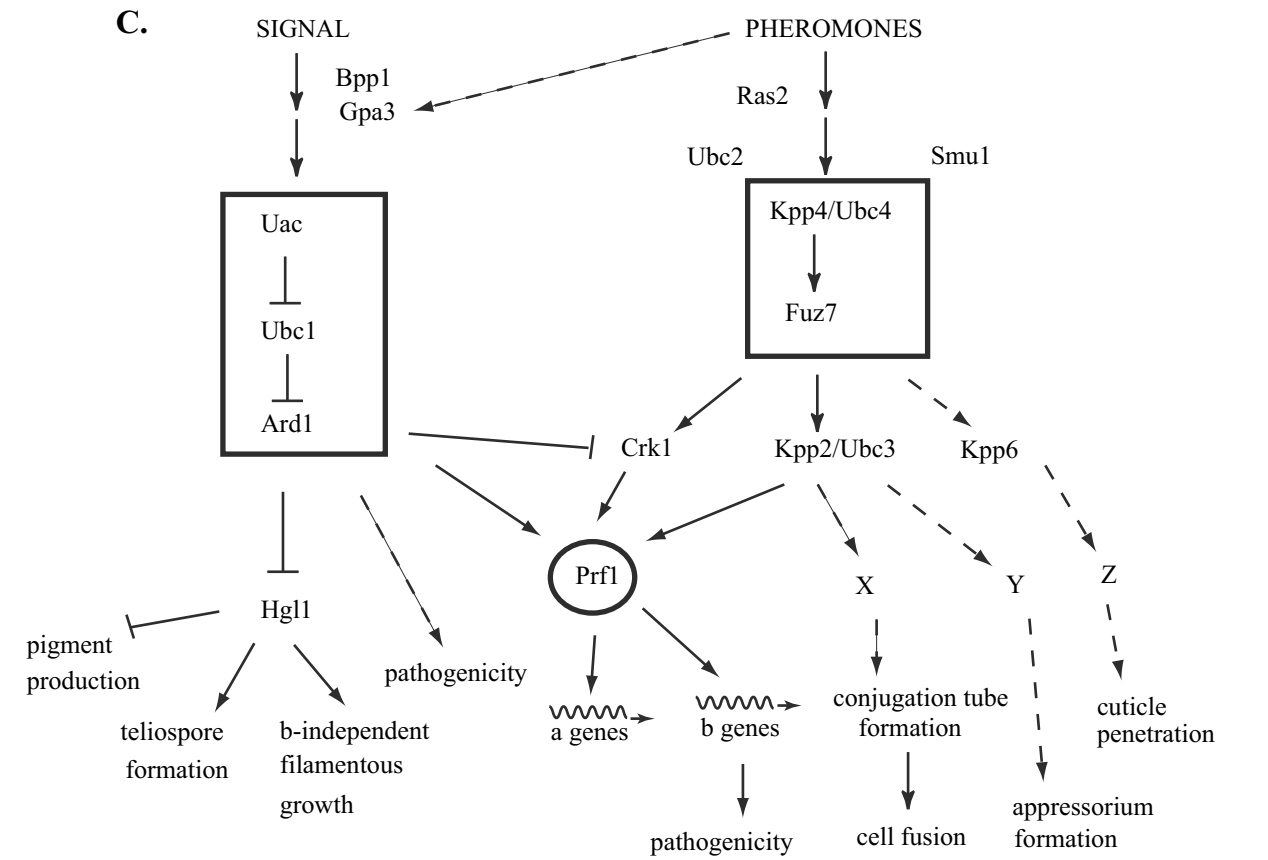
SIGNAL TRANSDUCTION PATHWAYS

The Pheromone Response Pathway

In *U. maydis*, as in other fungi, the pheromone signal is perceived by a cell surface receptor and transduced via a MAPK signal transduction pathway resulting in activation of a transcriptional activator (Fig. 5). The *U. maydis* receptors belong to the heterotrimeric G-protein-coupled receptors (GPCRs), containing seven transmembrane domains (Bölker et al., 1992). Heterotrimeric G proteins consist of three subunits: Gα, Gβ, and Gγ. In other eukaryotes, it has been shown that upon activation of the receptor by a signal, exchange of GDP for GTP on the Gα subunit causes its dissociation from the Gβγ subunit. Either or both Gα-GTP or Gβγ can activate downstream targets. *U. maydis* contains four Gα subunits, but only that coded by *gpa3* has been shown to be involved in signal transduction, and in particular, Gpa3 acts in the cAMP pathway (Fig. 5) (see below) (Regenfelder et al., 1997). *U. maydis* contains only one gene (*bpp1*) coding for a Gβ protein. *bpp1* appears to be a component of the cAMP pathway (Fig. 5) (see below) (Müller et al., 2004). The characterization of the gene that codes for the Gγ subunit has not been reported. It remains to be determined how the signal from the activated receptor is transmitted to the MAPK module.

The MAPK cascade module is a highly conserved module that mediates transduction of signals generated at the cell surface to the nucleus (see chapter 5). It consists of three Ser/Thr protein kinases: MAPKKK/MEKK, MAPKK/MEK, and MAPK/ERK. Transduction of the signal involves sequential phosphorylation: MAPKKK phosphorylates and activates the dual-specificity Ser/Thr tyrosine kinase MAPKK, which in turn phosphorylates and activates a MAPK. Multiple MAPK cascade modules exist in eukaryotic cells, and a given MAPK module may be activated by different signals, resulting in different outputs. Furthermore, cross talk exists between different MAPK modules and between MAPK modules and the cAMP pathway. The genome of *U. maydis* contains coding information for three MAPK modules: the pheromone response, the cell wall integrity, and the high osmolarity (reviewed in García-Pedrajas et al., 2008), of which only the pheromone response MAPK pathway has been characterized. The pheromone response MAPK module consists of Ubc4/Kpp4 (MAPKKK), Fuz7/Ubc5 (MAPKK), and Ubc3/Kpp2 (MAPK) (Fig. 5) (Andrews et al., 2000; Banuett and Herskowitz, 1994a; Mayorga and Gold, 1999; Müller et al., 1999, 2003). Two additional MAPKs, Kpp6 and Crk1, are part of this module and control different outputs (Fig. 5) (Brachmann et al., 2003; Garrido et al., 2004). The role of this module in pathogenicity is described in "The Infectious Process: Dramatis Personae" below. Ubc4/Kpp4,


A.
PHEROMONES
Ras2
Ubc2
Smu1
Kpp4/Ubc4
Fuz7
Kpp2/Ubc3
MAPKKK
MAPKK
MAPK
Prf1
X
TRANSCRIPTION FACTOR
b genes
a genes
conjugation tube formation
cell fusion
B.
UNIDENTIFIED SIGNAL
Ras2
Ubc2
Kpp4/Ubc4
Fuz7
Crk1
Kpp2/Ubc3
Kpp6
Prf1
Y
Z
cell cycle
b genes
appressorium formation
cuticle penetration
filamentous growth
pathogenicity


C.
SIGNAL
PHEROMONES
Bpp1
Gpa3
Ras2
Ubc2
Smu1
Uac
Kpp4/Ubc4
Ubc1
Fuz7
Ard1
Crk1
Kpp2/Ubc3
Kpp6
Prf1
X
Y
Z
Hgl1
pigment production
pathogenicity
a genes
b genes
conjugation tube formation
cuticle penetration
teliospore formation
b-independent filamentous growth
pathogenicity
cell fusion
appressorium formation

Fuz7/Ubc5, Ubc3/Kpp2, and Kpp6 are required for conjugation tube formation and filamentous growth on charcoal agar. Crk1 is also required for filamentous growth on charcoal agar, but its role in conjugation tube formation was not reported. (For details on the effects of mutations on expression of pheromone genes and receptors, the reader is referred to the original publications.) In *S. cerevisiae*, Ste20 interacts with and activates Ste11, the MAPKKK in the pheromone response MAPK module (see chapter 5; reviewed by Klosterman et al., 2007). In *U. maydis*, Smu1, a Ste20 p21-activated protein kinase (PAK), is required for conjugation tube formation (Smith et al., 2004) and may be upstream of the MAPK module (Fig. 5). Ubc2, a protein of 833 amino acids that contains a SAM (sterile alpha motif), a RA (Ras association) domain, and two SH3 (Src homology 3) domains, appears to act upstream of the MAPK module (Fig. 5) (Klosterman et al., 2008; Mayorga and Gold, 2001). Ubc2 interacts with Ubc4/Kpp4 via the SAM domain. Because the Ubc2 SAM domain has similarity to that of *S. cerevisiae* Ste50, it has been proposed that it plays a similar role in *U. maydis* (Klosterman et al., 2008), that is, to remove Ubc4/Kpp4 autoinhibition. Ubc2 is required for conjugation tube formation and for filament formation on charcoal agar, and the SAM domain is required for this activity (Mayorga and Gold, 2001). Its role in pathogenicity is described in "Ubc2, an Adaptor Protein" below.

A common target of activated MAPKs is a transcriptional activator. In *U. maydis*, Prf1 is the target of the Ubc3/Kpp2 and Crk1 MAP kinases (Fig. 5) (Kaffarnik et al., 2003). Prf1 belongs to the high-mobility-group class of transcriptional activators and activates transcription of genes that contain pheromone response elements (PRES): *mfa1*, *mfa2*, *pra1*, *pra2*, *bW*, *bE*, *lga2*, and *prf1* itself (Hartmann et al., 1996). Prf1 is phosphorylated by protein kinase A (PKA) and MAPK, and these phosphorylations are necessary for its activity and to allow promoter discrimination (Kaffarnik et al., 2003). Thus, pheromone and cAMP pathways converge on Prf1 (Fig. 5). Another input on Prf1 is the active b protein, which downregulates *prf1* expression in a dikaryon. Nutritional inputs, such as carbon source and perhaps nitrogen limitation, also converge on Prf1 via the cAMP pathway (Fig. 2) (Hartmann et al., 1999).

The cAMP Pathway

In *U. maydis*, as in other eukaryotes, the cAMP pathway consists of adenylyl cyclase (designated Uac1 in *U. maydis*), which catalyzes conversion of ATP to cAMP (see chapter 5). cAMP activates cAMP-dependent PKA, a tetrameric protein consisting of two regulatory subunits (Ubc1) and two catalytic subunits (Adr1) (Dürrenberger et al., 1998; Gold et al., 1994). Uka1 is another catalytic subunit but plays only a minor role. In the absence of cAMP, the tetrameric complex is inactive; the regulatory subunit prevents the catalytic subunit from phosphorylating its substrates. When cAMP is present, it binds the regulatory subunit and causes a conformational change that releases the catalytic subunit, which is now free to phosphorylate its substrates (Fig. 5).

The cAMP pathway is involved in the dimorphic transition. Mutations in *uac1* or *ard1* result in *b*-independent filamentous growth, whereas mutations in *ubc1* result in formation of multiple buds and a cell separation defect. These observations indicate that a low level of cAMP promotes filamentous growth and inhibits budding growth, whereas a high level of cAMP inhibits filamentous growth and promotes budding (Dürrenberger et al., 1998; Gold et al., 1994, 1997). Gpa3, a Gα subunit described above, acts upstream of Uac1 and may activate it in response to nutritional signals and to pheromones (Fig. 5) (reviewed by Kahmann and Kämper, 2004). Genetic analysis suggests that *bpp1* (coding for Gβ) is upstream of *gpa3* (Müller et al., 2004). Whether Gpa3 and Bpp1 (see above) are components of the same heterotrimeric G protein remains to be determined. Several genes that appear to be part of the cAMP pathway have been identified: *hgl1*, *vtc4*, *sep3*, and *ukb1*. *vtc4* links cAMP and polyphosphate accumulation; *sep3* (coding for a septin) links cAMP with proper septum position and morphogenesis; and *ukb1* (coding for a Ser/Thr protein kinase) may act upstream of the cAMP pathway (Abramovitch et al., 2002; Boyce et al., 2005; 2006; Dürrenberger et al., 2001). The roles of these genes, as well as those of *uac1*, *ubc1*, *adr1*, and *gpa3* in pathogenicity, are described in "The Infectious Process: Dramatis Personae" below.

Cross talk between the cAMP pathway and the pheromone response pathway is evidenced by the facts that (i) signals from the cAMP and MAPK pathways converge on Prf1 and (ii) cAMP levels determine the level of expression of pheromone precursor and receptor genes (reviewed by Banuett, 2002; Kahmann and Kämper, 2004; and Klostermann et al., 2007).

The Response to Lipids: a Clue to Plant Signals?

In the presence of a variety of lipids, *U. maydis* cells undergo a dimorphic transition from yeast-like to filamentous growth, and this response depends on an intact MAPK module, a Ras2 protein, and the cAMP pathway (Klose et al., 2004). As described in "The Infectious Process: a Drama in Five Acts" above, the filamentous morphology induced by lipids is similar to that observed in planta. This is

FIGURE 5 Signal transduction in *U. maydis*. The MAPK and cAMP signal transduction pathways regulate formation of the infectious filamentous dikaryon and also interaction with the host plant. (A) Pheromones activate a MAPK module consisting of Kpp4/Ubc4, Fuz7/Ubc5, Kpp2/Ubc3, and Crk1. Both Kpp2 and Crk1 activate Prf1, which in turn binds to pheromone response element sites present in the upstream regions of genes in the *a* locus (*mfa1*, *mfa2*, *pra1*, *pra2*, and *lga2*) and the *b* locus (*bW* and *bE*). Activation of the *b* genes in the haploid ensures that upon fusion of haploid cells containing different *b* alleles, the active b protein is readily formed and activates the filamentous program and pathogenic development. In addition, Kpp2/Ubc3 acts via an unknown transcriptional activator to control formation of conjugation tubes, which mediate cell fusion. Ubc2, an adaptor protein, interacts with Kpp4/Ubc4. Smu1 (Ste20) is likely upstream of the MAPK module, though this remains to be determined. Ras2 is proposed to act upstream of the MAPK module (see the text). (B) Once the filamentous dikaryon is formed in the plant, the same MAPK module and another MAPK (Kpp6) in response to putative plant signals activate appressorium formation (Kpp2/Ubc3), cuticle penetration (Kpp6), and filamentous growth and pathogenicity. (C) The signals that activate the cAMP and pheromone response MAPK pathways converge on Prf1. Prf1 is phosphorylated by the MAPKs Kpp2/Ubc3 and Crk1 and by Adr1, the catalytic subunit of PKA. Gpa3 activates the cAMP pathway in response to pheromones and to nutritional inputs, including lipids and phosphate (see the text). Components of the cAMP pathway play roles at various stages of the infectious cycle (see the text).

the first demonstration of induction of hyphal differentiation in culture that mimics the one induced in planta. These studies indicate that lipids may be an important plant signal required for differentiation and growth of the *b*-dependent filamentous form during infection. Alternatively, lipids may simply serve as a carbon source during growth in the plant (Klose et al., 2004).

THE INFECTIOUS PROCESS: DRAMATIS PERSONAE

In this section, the roles of different genes in the infectious process are presented (Table 1). Several genes appear to act early, others late, and still others at multiple steps in the infectious process. It is important to keep in mind that a mutation that blocks an early step could also block later steps. Future analysis in which genes are expressed conditionally will likely provide insights about the requirement of each gene throughout the infectious process. The reader is referred to the original literature for additional interesting observations.

Formation of the Infectious Dikaryon

The *b* Locus and the Pheromone MAPK Module

The *b* locus is critical for formation of the dikaryotic filament, and *b* is the earliest-acting gene in the infectious process (Table 1), barring genes required for cell fusion and dikaryon establishment. Inoculation of plants with strains carrying identical *b* alleles, for example, diploids heterozygous at *a* and homozygous at *b* such as *a1/a2 b1/b1* or a mixture of *a1 b1* and *a2 b1* haploids, results in the formation of short, distorted multinucleate filaments that do not grow extensively and degenerate, similar to those observed in culture (see "The *a* Locus" above) (Banuett and Herskowitz, 1994a, 1994b, 1996; Snetselaar, 1993; Snetselaar and Mims, 1994). The *a* locus is dispensable for pathogenicity as shown by inoculation of plants with strains that bypass the requirement for cell fusion, such as diploids homozygous at *a* and heterozygous at *b*, for example, *a1/a1 b1/b2* or haploids containing two different *b* alleles and one *a* allele, for example, *a1 bW1 bE2* (Banuett and Herskowitz, 1996; Regenfelder et al., 1997). A recent observation indicates that in special circumstances, different *a* alleles are required for filament formation in planta. If strains contain different *b* alleles and only one *a* allele as well as a deletion of the *bpp1* gene (*a1 bW1 bE2 Δbpp1*), which codes for a Gβ subunit, they do not form filaments in the plant (Müller et al., 2004); if the strains contain different *a* alleles, *bpp1* is dispensable for filament formation in planta (Müller et al., 2004). These observations suggest that *bpp1* is important for transmission of a signal necessary for filament formation in the absence of different *a* alleles.

Components of the pheromone response MAPK module, Ubc4/Kpp4, Fuz7/Ubc5, Ubc3/Kpp2, and Crk1 (see "The Pheromone Response Pathway" above), play a role early in infection: they control cell fusion and filamentous growth of the infectious dikaryon. They also exert a role independently of cell fusion (and the *a* locus) in filamentous growth and pathogenicity (Andrews et al., 2000; Banuett and Herskowitz, 1994a; Müller et al., 2003). This observation led to the hypothesis that the MAPK module is activated in response to plant signals. One type of signal may be plant lipids (see "The Infectious Process: a Drama in Five Acts" and "The Response to Lipids: a Clue to Plant Signals" above) (Klose et al., 2004).

cAMP and Early Steps in Infection

cAMP plays a key role in pathogenic development of *U. maydis* and other fungi (reviewed by Lee et al., 2003). In *U. maydis*, analysis of mutations in different genes in the pathway has led to the conclusion that altered levels of cAMP interfere with tumor induction and teliospore development (Krüger et al., 2000). Null mutations in *gpa3*, *uac1*, or *adr1* result in filamentous growth of haploids in culture, but these strains are not capable of inducing tumors in pure culture, indicating that this filamentous program differs from that activated by *b*. Comparison of expression profiling patterns of these pathways would likely uncover genes that are specific for each pathway.

Inoculation of plants with *Δgpa3*, *Δuac1*, or *Δadr1* strains of opposite mating type does not result in symptoms (Table 1). The specific block was not described, but the lack of any symptoms, including chlorosis, suggests involvement in very early steps, such as formation of the infectious dikaryon or development of appressoria (Dürrenberger et al., 1998; Gold et al., 1994; Regenfelder et al., 1997). Inoculation of plants with a mixture of *Δadr1* mutants and wild-type strains of opposite mating type results in reduced tumor formation compared to inoculations with wild-type strains, suggesting reduced ability to mate and consequently to form the infectious dikaryon. Diploids heterozygous at the mating-type loci and homozygous for the *Δadr1* mutation did not induce symptoms, indicating that *adr1* is necessary for filamentous growth per se, independently of any role it may have on cell fusion and establishment of the dikaryon (Dürrenberger et al., 1998). Whether the *adr1* null mutants are able to form appressoria, penetrate or proliferate in the plant, or block even earlier steps remains to be determined. *uka1* codes for a minor catalytic subunit of PKA that is dispensable for pathogenicity (Dürrenberger et al., 1998). The effect of mutations in *gpa3* and *ubc1* on teliospore development is described in "Components of the cAMP Pathway: *gpa3* and *ubc1*" below.

Penetration of Host Cells: Formation of Appressorium-Like Structures

The *biz1* Gene

The *biz1* gene was identified in a screen to isolate mutants altered in cell cycle arrest. It codes for a protein of 783 amino acids that contains a C_2H_2 zinc finger domain and is required for formation of appressorium-like structures (Flor-Parra et al., 2006). Inoculation of plants with *Δbiz1* mutants (*a1 b1 Δbiz1 + a2 b2 Δbiz1*) results in filament formation on the leaf surface, but few appressoria develop (Table 1) (Flor-Parra et al., 2006). In the rare cases where appressoria are formed, the filaments are able to penetrate but do not proliferate. The filaments formed upon cell fusion of *a1 b1 Δbiz1 + a2 b2 Δbiz1* in culture contain two or more nuclei, which was interpreted as evidence of release from cell cycle arrest (Flor-Parra et al., 2006). The *biz1* gene is expressed on charcoal media in a b-dependent manner. The absence of *bbs* sites in its upstream regulatory region suggests that it is an indirect target of *b* (Brachmann et al., 2003). *biz1* is expressed in planta from 2 to 13 days postinfection (dpi), which may indicate that Biz1 is required at different stages during the infectious process, though this remains to be determined.

The Pheromone Response MAPK Module: Ubc4/Kpp4, Fuz7/Ubc5, Ubc3/Kpp2, and Kpp6

The *ubc4/kpp4*, *fuz7/ubc5*, and *ubc3/kpp2* genes are required for appressorium formation (Table 1). Mutants altered in these genes form few filaments in planta, and those that are

TABLE 1 Genes required for pathogenicity

Gene	MIPS um no.[a]	Protein function	Role in the interaction
b			
bE1	12052	Homeodomain polypeptide	Key determinant of pathogenicity. Required for filamentous
bW1	00578	Homeodomain polypeptide	growth, pathogenicity, and meiosis
adr1	04456	Catalytic subunit of cAMP-dependent protein kinase	Required for tumor induction; probably acts early in infection
biz1	02549	C_2H_2 zinc finger	Required for formation of appressoria and proliferation
brh2	03200	BRCA2 homologue	Teliospore germination: formation of the promycelium
cdk5	11892	Pho85 orthologue	Full tumor response
chs6	10367	Chitin synthase	Proliferation
chs7	05480	Chitin synthase	Full tumor response
clip1	06338	Similarity to *Coprinopsis cinerea* Clip1	Formation of clamp-like structures; proliferation
clb2	10279	B-type cyclin	Tumor induction. Hyphal morphology in planta
crk1	11410	MAPK	Full tumor response and teliospore formation
cru1	03917	APC-like	Tumor induction; proliferation; normal teliospore morphology, and teliospore germination. Hyphal morphology in planta
fer1	00105	Fe transport multicopper oxidase	Full tumor response and teliospore formation
fer2	10023	Similarity to high-affinity Fe permeases	Required for proliferation; full tumor response; teliospore formation
fuz1	02587	MYND domain zinc finger	Full tumor response. Teliospore development; required for hyphal fragmentation
fuz2	ND	Uncharacterized	Teliospore germination
fuz7/ubc5	01514	MAPKK	Formation of appressoria; teliospore germination
gas1	04405	ER glucosidase II	Induction of anthocyanin; integrity of interaction zone; proliferation
gpa3	04474	Gα	Required for tumor induction and for teliospore formation, most likely at the cell-rounding stage after hyphal fragmentation.
glo1	02411	Similarity to glyoxal oxidase from *P. chrysosporium*	Required for tumor induction. Specific step not determined
hda1	02065	Similarity to histone deacetylase	Required for teliospore formation, likely at the cell-rounding or hyphal fragmentation stage
hgl1	11450	No similarities to proteins in the databases	Required for teliospore formation, after hyphal fragmentation
khd4	03837	RNA binding protein with K homology domains	Full tumor response; specific step altered not described
kpp2/ubc3	03305	MAPK	Formation of appressorium-like structures
kpp4/ubc4	04258	MAPKKK	Formation of appressorium-like structures
kpp6	02331	MAPK	Required for penetration; induction of anthocyanin
mfe2	10038	Similarity to multifunctional b-oxidation enzyme	Hyphal branching; full tumor response; development of teliospores
mcs1	03204	Myosin-chitin synthase	Proliferation; hyphal morphology in the plant
mrb1	06182	Mitochondrial protein of the p32 family	Required for hyphal branching and proliferation
myo5	04555	Type V myosin	Hyphal morphology in planta; proliferation; full tumor response
myp1	05842	No similarities to proteins in databases	Tumor induction; step altered not described
pcl12	02712	Cyclin of the Pcl1,2 subfamily	Hyphal morphology in planta; teliospore germination
rad51	03290	Rad51 homologue	Teliospore germination: formation of the promycelium
ras2	01643	GTP-binding protein	Required for pathogenicity; step altered not described
rec2	03095	Divergent Rad51 paralog	Basidiospore formation on the promycelium

(Continued on next page)

TABLE 1 Genes required for pathogenicity (*Continued*)

Gene	MIPS um no.[a]	Protein function	Role in the interaction
rrm4	10836	RNA binding protein with RRM domains	Full tumor response; specific step altered not described
rum1	02582	Similarity to human retinoblastoma binding protein	Required for teliospore formation, likely at the cell-rounding or a prior step
sep3	03449	Septin; CDC11-like	Full tumor response; probably formation of teliospore cell wall; normal morphology of the promycelium
smu1	12272	PAK; similar to *S. cerevisiae* Ste20	Full tumor response; specific step altered not described
uac1	05232	Adenylyl cyclase	Required for tumor formation; likely acts early in infection
ubc1	00525	Regulatory subunit of PKA	Required for tumor induction and teliospore formation, likely at the cell rounding stage after hyphal fragmentation
ubc2	05261	Adaptor protein	Tumor induction; specific step altered not described
ukb1	03315	Ser/Thr protein kinase	Hyphal branching and proliferation; tumor induction
ukc1	04956	Ser/Thr protein kinase homologous to Cot-1 and Orb6	Tumor induction; step altered not described
yap1	02191	Basic leucine zipper transcription factor	Full tumor response
yup1	05406	Early endosomal t-SNARE	Hyphal morphology in planta; teliospore formation, most likely after hyphal fragmentation; teliospore germination; morphology of promycelium.
vtc4	10968	Homologous to yeast VTC4 encoding vacuolar transport chaperone 4	Proliferation and full tumor response
	10672	Similarity to heme peroxidase	Full tumor response
	01947	Similarity to cytochrome *c* peroxidase precursor	Full tumor response
Cluster 2A	01234–01240	Mostly, conserved hypothetical *Ustilago*-specific proteins	Hypervirulent: number and size of tumors increased
Cluster 6A	02533, 02535 02537, 02538, 02540, 11415–17	Mostly, conserved hypothetical *Ustilago*-specific proteins	Required for a full tumor response
Cluster 10A	03644–03753	Mostly, conserved hypothetical *Ustilago*-specific proteins	Required for a full tumor response
Cluster 19A	05294–05296, 05299–05303, 05305–05306, 05308–05314, 05316–05319, 10553–10557	Mostly, conserved hypothetical *Ustilago*-specific proteins or hypothetical proteins	Full tumor response and formation of teliospores
Cluster 5B	02473–02475	Conserved hypothetical *Ustilago*-specific proteins or hypothetical proteins	Required for penetration

[a]MIPS, Munich Information Center for Protein Sequence.

formed lack appressoria. These two phenotypes likely explain the lack of symptom development in plants inoculated with *Δfuz7/ubc5* or *Δubc4/kpp4* strains carrying different *a* and *b* alleles (Andrews et al., 2000; Banuett and Herskowitz, 1994a; Mayorga and Gold, 1999; Müller et al., 1999; 2003). Fuz7/Ubc5 is also required for teliospore germination (Table 1) (Banuett and Herskowitz, 1994a, 1994b). Whether other components of the pheromone response MAPK are required for teliospore germination remains to be determined.

Inoculation of plants with *Δkpp2/Δubc3* null mutants (*a1 b1 Δkpp2/Δubc3* + *a2 b2 Δkpp2/Δubc3*) reduces tumor formation compared to inoculation with wild-type strains (9% for the mutants versus 95% for the wild type), and further reduction occurs in inoculations with strains of opposite mating type carrying a mutation that results in a kinase-dead protein (3%), indicating that another MAPK can take the place of the absent MAPK. This other MAPK is Kpp6 (Mayorga and Gold, 1999; Muller et al., 1999, 2003). Inoculation of plants with *Δkpp6 Δkpp2* double-mutant strains (*a1 b1 Δkpp6 Δkpp2* + *a2 b2 Δkpp6 Δkpp2*) completely abolishes residual tumor formation, supporting the notion that these two MAPKs have partially redundant functions. The targets of Kpp6 are unknown; one target of Ubc3/Kpp2 is Prf1.

Kpp6 is required for penetration and induction of anthocyanin pigmentation (Table 1) (Brachmann et al., 2003).

Infection with *Δkpp6* strains (*a1 b1 Δkpp6* + *a2 b2 Δkpp6*) does not impair appressorium formation, but penetration is severely affected. The fact that no anthocyanin pigmentation develops indicates that Kpp6 is required for its induction. Despite its defect in penetration, *Δkpp6* mutants form tumors, likely reflecting functional redundancy with Kpp2/Ubc3, albeit the response is reduced and the tumors are smaller. The tumors produce teliospores that germinate normally. Interestingly, it was reported that the infected plants are taller and heavier (Brachmann et al., 2003). The *kpp6* gene is regulated by the *b* locus, but most likely indirectly because *kpp6* does not contain a *bbs* site (Brachmann et al., 2003).

Proliferation: Nuclear Partitioning, Branch Formation, and Tumor Induction

The filaments inside plant cells are surrounded by the plasma membrane of the host cell (Snetselaar and Mims, 1994), and this intimate contact between hyphal tip and plant cell membrane (the interaction zone; Bauer et al., 1997) may be the portal for exchange of signals and metabolites (see "Clamp-Like Structures, Fungal Proliferation within Host Cells, and Tumor Induction" above).

Myo5, a Type V Myosin

myo5 codes for a type V myosin of 1,853 amino acids. *myo5* is required for normal cell morphogenesis, conjugation tube formation, and filamentous growth on charcoal agar (Weber et al., 2003). A dikaryon generated upon cell fusion between *a1 b1 myo5^{ts}* and *a2 b2 myo5^{ts}* exhibits irregular growth, although polarized growth is not altered (Weber et al., 2003). Inoculation of plants with *myo5^{ts}* strains results in formation of few dikaryotic filaments. These hyphae are capable of penetration, and once inside the plant cells they develop multiple swollen branches (Table 1) (Weber et al., 2003). In plant inoculations with *myo5^{ts}* strains, tumor formation is reduced at 22°C (2.7% for the mutant versus 98.9% for the wild type) and abolished at 28°C (compared to 82% for the wild type) (Table1). Myo5::GFP (green fluorescent protein) localizes to hyphal tips and septa and displays a punctate pattern in the maturing teliospore.

Gas1, an ER Endoglucosidase

gas1 was identified using restriction enzyme-mediated insertional mutagenesis (REMI) in a screen for nonpathogenic mutants. *gas1* codes for a glucosidase II α-subunit that contains an amino-terminal signal sequence and localizes to the endoplasmic reticulum (ER) (Schirawski et al., 2005). *gas1* null mutants have no effect on filament formation on charcoal agar, although they exhibit increased cell wall staining in yeast-like and hyphal cells. Inoculation of plants with *Δgas1* strains of opposite mating type abolishes anthocyanin pigmentation and tumor formation (Table 1). Examination of plant tissue shows that the mutant forms appressoria and is able to penetrate but proliferation arrests soon after penetration. Fungal hyphae do not extend deeper than the epidermis layer. Thus, *gas1* plays a pivotal role in the ability of the fungus to invade different layers of the leaf. *gas1* mutant hyphae exhibit irregular deposits of calcofluor-staining material and appear to be defective in complete translocation of the cytoplasm from the appressorial cell to the hyphal part inside the plant cell. In addition, the region around the interaction zone is aberrant (Schirawski et al., 2005).

Ubc2, an Adaptor Protein

ubc2 codes for an adaptor protein (see "The Pheromone Response Pathway" above) and is required for tumor induction (Table 1). *ubc2* mutants form intracellular hyphae that proliferate but cannot induce tumors (Mayorga and Gold, 2001). The SH3 domains and the carboxy-terminal region are required for pathogenicity (Klosterman et al., 2008).

Components of the Cell Cycle Machinery: a Cyclin and APC

clb2 codes for a B-type cyclin that is essential for viability (García-Muse et al., 2003b). Inoculation of plants with a wild-type strain and a strain expressing *clb2* constitutively abolishes tumor induction: 66% of plants exhibit no symptoms, and the remaining exhibit chlorosis only; there is no anthocyanin production (Table 1). The hyphae formed in these inoculations develop more branches than those obtained with wild-type strains and are able to proliferate in plant tissues but do not induce tumors (Table 1) (García-Muse et al., 2003b). An *a1/a2 b1/b2 Δclb2/clb2* diploid strain forms filaments on charcoal agar in a fashion similar to that of an *a1/a2 b1/b2 clb2/clb2* diploid strain; however, these strains differ in pathogenicity. The former strain induces chlorosis and anthocyanin pigmentation but no tumors, whereas the latter produces a normal tumor response. This apparent haploinsufficiency is also evident in the morphology of the hyphae, which are wider, less septate, and with fewer branches than those formed by wild-type strains (García-Muse et al., 2003b).

The *cru1* (cell cycle regulator) gene codes for a protein of 592 amino acids that belongs to the APC family of cell cycle regulators (Castillo-Lluva et al., 2004). Ectopic expression of *cru1* induces a G_2 cell cycle arrest in culture. *cru1* mRNA levels are increased in *ubc1* mutants and decreased in *adr1* mutants. Thus, PKA appears to regulate the level of expression of *cru1*. Inoculation of plants with *Δcru1* strains of opposite mating type results in tumor formation in only 5% of the plants, compared to 90% in inoculations with wild-type strains. The tumors are smaller and contain fewer teliospores. The teliospores are irregularly shaped and unable to germinate (Table 1) (Castillo-Lluva et al., 2004). Chlorosis was observed in 38% of the plants that did not develop tumors. Microscopic examination of these areas indicated that mutant hyphae were shorter (containing four to five cell compartments) than wild-type hyphae. These observations indicate that *cru1* is necessary for different steps of the infectious cycle: hyphal morphology and proliferation, formation of normal teliospores, and teliospore germination (Table 1) (Castillo-Lluva et al., 2004).

Clamp-Like Structures and Nuclear Partitioning: Clip1

The *clip1* gene was identified using mRNA profiling analysis of *b*-regulated genes (Scherer et al., 2006). *clip1* contains two *bbs* sites and is thus likely a direct target of the b protein. It codes for a protein of 444 amino acids that exhibits similarities to Clip1 of *C. cinereus*, two putative Clip1-like proteins in *Phanerochaete chrysosporium*, and one Clip1-like protein in *C. neoformans*. *U. maydis clip1* null mutants fail to induce tumors. Examination of infected tissue indicates that appressorium-like structures are formed and penetration is not impaired but proliferation fails. The hyphae lack clamp-like structures, which are necessary for nuclear partitioning (Table 1) (see "Clamp-Like Structures, Fungal Proliferation within Host Cells, and Tumor Induction" above) (Scherer et al., 2006). These observations indicate that formation of clamp-like structures and proper nuclear distribution are required for proliferation (Scherer et al., 2006).

A Clip1::3xeGFP protein fusion localizes to the nucleus. Although the *clip1* gene appears to be expressed prior to penetration, protein localization occurs after penetration, suggesting posttranscriptional or posttranslational modification. Expression of *clip1* in the plant peaks at 7 dpi (Scherer et al., 2006). Overexpression of *clip1* (approximately six times more than normal) blocks *b*-dependent filament formation in culture. The authors concluded that overexpression of *clip1* releases G_2 arrest, though this was not confirmed by fluorescence-activated cell sorter (FACS) analysis, and expression of several *b*-dependent genes was downregulated (Scherer et al., 2006). These observations suggest that *b* and *clip1* may regulate each other. One possible scenario is that *b* activates *clip1* gene expression, and once Clip1 reaches a certain threshold level, Clip1 downregulates *b*, perhaps controlling b stability or activity. Clip1 does not appear to be a DNA binding protein (Scherer et al., 2006).

The cAMP Pathway and Proliferation: *ubc1* and *ukb1*

Inoculation of plants with *Δubc1* strains of opposite mating type results in early symptom development (anthocyanin production) but no tumors, though the mutant is able to form hyphae that proliferate in the host cells (Table 1) (Gold et al., 1994, 1997). Ubc1 is required for filamentous growth on charcoal agar: *ubc1* mutants attenuate the response, suggesting that high levels of cAMP are partially inhibitory to filament formation in culture. Because *ubc1* mutants form hyphae in the plant, these observations indicate that high levels of cAMP do not interfere with filament formation in planta but rather with the ability of hyphae to induce tumors.

The *ukb1* gene codes for a Ser/Thr protein kinase of 1,216 amino acids that exhibits greater similarity to PKB than to PKA or PKC. In addition to the kinase domain, Ukb1 contains other motifs in the amino- and carboxy-terminal regions that flank the kinase domain, which suggests a role in morphogenesis, for example, talin and tubulin binding domains (Abramovitch et al., 2002). Ukb1 appears to inhibit lateral bud formation: *ukb1* mutants form lateral buds (40% in the mutant compared to 2% in wild-type strains). In addition, *ukb1* is necessary for tumor induction: *Δukb1* strains of opposite mating type do not induce tumor but are able to form dikaryotic hyphae that proliferate in plant tissues, though their growth is not as extensive as that of wild-type hyphae (Table 1). Furthermore, the number of branches and small buds increases in *ukb1* mutant hyphae compared to wild-type hyphae (Abramovitch et al., 2002). The predicted Ukb1 protein contains 30 potential phosphorylation sites for PKA and 25 such sites for PKC and is perhaps a target of these protein kinases. The *Δukb1* mutant does not suppress the filamentous phenotype of an *adr1* mutant and was thus proposed to act upstream of PKA or in a parallel pathway (Abramovitch et al., 2002).

Iron Uptake: the *fer* Genes, a Link to cAMP

Prf1 is a well-characterized target of the cAMP pathway (see "The Pheromone Response Pathway" above). Other potential targets include Ukb1 (described in "The cAMP Pathway and Proliferation: *ubc1* and *ukb1*" above) and Hgl1 (described in "A Putative Target of PKA: Hgl1" below). Expression profiling of genes regulated by overexpression of *adr1* indicates that 847 genes are upregulated; 407 of these appeared to be coregulated and include a group of three unlinked gene clusters involved in iron uptake (Eichorn et al., 2006). One of these clusters contains the *sid1* and *sid2* genes involved in siderophore biosynthesis, previously described by S. A. Leong and colleagues (see Yuan et al., 2001, and references therein). In the presence of high levels of iron, both genes are repressed by Urbs1, a GATA regulatory protein (Yuan et al., 2001, and references therein). *sid1*, *sid2*, and *urbs1* are not required for pathogenicity (Yuan et al., 2001, and references therein). Another gene cluster contains eight genes predicted to have a role in iron uptake, designated *fer* (Fe regulated) genes (for putative functions of these genes, see Eichhorn et al., 2006). The upstream region of the *fer* genes contains Urbs1 binding sites. All *fer* genes are induced under low iron conditions and repressed in medium containing 10 μM $FeSO_4$, in an Urbs1-dependent manner (Eichhorn et al., 2006). Expression of the genes in this cluster requires the cAMP pathway: no or reduced RNA was observed in *Δuac1* mutants. *fer2*, one of the genes in this cluster, codes for a protein of 486 amino acids with similarity to other fungal high-affinity iron permeases. *Δfer2* mutants exhibit reduced growth on low-iron medium. Inoculation of plants with *fer2* null mutants (*a1 b1 Δfer2* + *a2 b2 Δfer2*) reduces pathogenicity: appressoria are formed, but proliferation is reduced. The percentage of tumors is also reduced, and few teliospores are formed (Table 1) (Eichhorn et al., 2006). *fer1* null mutants also have attenuated pathogenicity: tumor induction and teliospore formation were reduced (Table 1). Although *fer1* and *fer2* null mutants exhibit reduced production of teliospores, they are not arrested in the teliospore developmental program. Analysis of *fer1* and *fer2* genes highlights the importance of iron acquisition for fungal proliferation in the plant.

Fer2::eGFP localizes to the plasma membrane in yeast-like cells in culture and in hyphae in planta prior to sporogenesis but is not detected at later stages. The fusion protein was not detected at earlier stages of growth in the plant (Eichorn et al., 2006).

Phosphate Acquisition: *vtc4* and a Link to cAMP

Serial analysis of gene expression (SAGE) led to the identification of transcripts whose expression varies with PKA activity (high in *ubc1* mutant libraries and low in *adr1* mutant libraries) (Larraya et al., 2005). Expression of several genes encoding ribosomal proteins, metabolic functions (including peptide transporters and a putative sugar transporter), morphogenesis (*sep3*), a repellent protein, prefoldin, cell wall synthesis, and phosphate acquisition (*vtc4*) varied depending on PKA activity (Larraya et al., 2005).

The *vtc4* gene codes for a predicted protein of 833 amino acids containing three putative transmembrane domains and an amino-terminal SPX domain, which is found in proteins involved in G-protein-dependent signal transduction or that sense phosphate levels (Boyce et al., 2006). *vtc4* is homologous to *S. cerevisiae VTC4*, a gene encoding vacuolar transport chaperone 4. Its transcript is increased in *ubc1* mutants, and thus, Vtc4 may be a target of Adr1 (Boyce et al., 2006). The *Δvtc4* mutation does not interfere with formation of conjugation tubes or filaments on charcoal agar. Inoculation of seedlings with *Δvtc4* mutants of opposite mating type reduces the tumor response, probably due to reduced fungal proliferation (Table 1). The tumors that form contain teliospores, whose germination is not affected. Inoculation of silks with the *Δvtc4* mutant results in a degree of kernel infection similar to that observed with wild-type strains (Boyce et al., 2006). Thus, *vtc4* is required for a full tumor response in seedlings but is dispensable in kernel infections. The observed differences in tumor response led the authors to conclude that nutrient availability differs in seedlings versus

kernels or that the path to the target tissue may be more direct via silk inoculation than via seedling inoculation (Boyce et al., 2006). The *vtc4* gene is also required for accumulation of polyphosphate in the vacuole. *Δvtc4* may provide an important link between nutrient acquisition in the plant and the cAMP pathway (Boyce et al., 2006).

Oxidation of Fatty Acids: *mfe2*

The *mfe2* gene was isolated based on similarity to *S. cerevisiae FOX2*, which codes for a multifunctional enzyme involved in β-oxidation (Klose and Kronstad, 2006). The *mfe2* gene codes for a predicted protein of 911 amino acids that contains two dehydrogenase and one hydratase domains. Deletion of *mfe2* does not affect conjugation tube or dikaryotic filament formation on charcoal agar or in the plant. The *mfe2* null mutants are not able to exhibit lipid-induced filamentous growth (Klose and Kronstad, 2006). Inoculation of plants with *Δmfe2* strains of opposite mating type results in reduced tumor response: 27% of the plants developed tumors compared to 88% of plants inoculated with wild-type strains (Table 1). Microscopic examination of infected tissue indicates that the mutants are able to form the filamentous dikaryon and penetrate, but hyphal branching is delayed compared to that observed in inoculations with wild-type strains. Teliospore development is also delayed, but teliospore germination is not affected. These observations indicate that *mfe2* is required for extensive branching of the filamentous dikaryon, which likely affects the ability of the fungus to proliferate and induce tumors (Klose and Kronstad, 2006).

The Crk1 MAPK

The *crk1* gene codes for a MAPK (Garrido et al., 2004) required for transcription of Prf1, the pheromone response transcriptional activator (see "The Pheromone Response Pathway" above). Phosphorylation of Crk1 by Fuz7 is necessary for its activity. Crk1 and Fuz7/Ubc5, as well as Crk1 and Ubc3/Kpp2, physically interact (Garrido et al., 2004). *Δcrk1* mutants abolish filament formation on charcoal agar. Because the *Δcrk1* mutation also abolishes filamentous growth in a strain that bypasses the need for cell fusion, it indicates that *crk1* is required for postfusion events, in addition to any requirements for cell fusion (Garrido et al., 2004). Inoculation of plants with *Δcrk1* strains of opposite mating type results in reduced tumor response (Table 1): approximately 8% of the plants develop tumors, which lack teliospores, indicating that *crk1* is necessary for a full tumor response and for teliospore production (Garrido et al., 2004). The steps altered during the infectious process were not described. A *Δcrk1* mutation suppresses the filamentous phenotype of an *adr1* mutant (see Garrido et al., 2004, for reference), as do mutations in other components of the pheromone response MAPK module (see Mayorga and Gold, 1999, and references therein).

Chitin Synthases: Chs6, Chs7, and Mcs1

U. maydis contains eight chitin synthases: *chs1* through *chs7* and *mcs1*, a hybrid myosin-chitin synthase specific to the filamentous fungi (Weber et al., 2006). Inoculation of plants with strains of opposite mating type deleted for each of the chitin synthase genes indicates that *chs6*, *chs7*, and *mcs1* are necessary for tumor induction, whereas *chs1* through *chs5* play only a minor role (Table 1) (Garcerá-Teruel et al., 2004; Weber et al., 2006). The defect of the *chs7* null mutant appears to be due in part to a defect in cell fusion: a solopathogenic *chs7* mutant strain that bypasses the cell fusion requirement is able to induce tumors, though at a reduced rate compared to wild-type strains (Weber et al., 2006). It was not reported if fungal proliferation was altered in inoculations with *chs7* null mutants. *Δchs6* strains form hyphae that penetrate, but fungal mass is reduced, indicative of reduced proliferation resulting in the absence of tumors (Garcerá-Teruel et al., 2004). *Δmcs1* strains form infectious filaments that are able to penetrate the epidermis. Once inside this layer, the hyphal cells become swollen, indicative of loss of polarized growth, and the hyphae are not able to proliferate. Taken together, these observations indicate that at least three chitin synthases are necessary for fungal proliferation and a full tumor response (Weber et al., 2006).

A Cot-1 and Orb6 Homologue: Ukc1

The *ukc1* gene codes for a Ser/Thr protein kinase of 608 amino acids belonging to the cAMP protein kinase subfamily (Dürrenberger et al., 1999). Ukc1 is homologous to *S. pombe* Orb6 and *Neurospora crassa* COT-1, both of which are required for normal cell morphogenesis in these fungi. *ukc1* is required for normal cell morphology and to inhibit production of a dark pigment: *ukc1* null mutants form clusters of rounded, darkly pigmented cells with long appendages. The pigment may be melanin. Similar morphology has been described for wild-type strains under conditions of low pH (Ruiz-Herrera et al., 1995). *a1/a2 b1/b2 ukc1*$^{-}$*/ukc1*$^{-}$ diploids are unable to form filaments on charcoal agar, indicating that Ukc1 is required for filamentous growth independently of any requirement for cell fusion. *ukc1* is also required for pathogenicity: inoculation of plants with *Δukc1* mutants of opposite mating type results in anthocyanin pigmentation but no tumor induction. The step altered by the *ukc1* mutation was not described (Table 1).

Gene Clusters for Putative Secreted Proteins: Effectors of Tumor Induction or Antagonists of Plant Defense?

Inspection of the *U. maydis* genome indicates that it does not contain coding information for the extensive battery of cell wall-degrading enzymes possessed by necrotrophic pathogenic fungi, such as *M. grisea* and *Fusarium graminearum* (Kämper et al., 2006). The argument has been made that this reflects the biotrophic lifestyle of *U. maydis*, in which integrity of host tissues is preserved for most of the infectious cycle. In contrast to the scarcity of hydrolytic enzymes, *U. maydis* contains an unusually large number of predicted small secreted proteins of unknown function, of which two-thirds appear to be specific to *U. maydis* (Kämper et al., 2006). Of the genes predicted to code for secreted proteins, 18.6% are found in 12 gene clusters, dispersed throughout the genome, and the clusters contain 3 to 26 genes. Eight of the 12 clusters contain two to five related genes in tandem arrays and are predicted to have arisen by gene duplication (Kämper et al., 2006). Most genes in the clusters are induced in the tumors, whereas flanking genes are not, suggesting a possible role during infection (Kämper et al., 2006). Deletion analysis of each individual cluster indicates that seven clusters (1A, 2B, 3A, 5A, 8A, 9A, and 22A) are not essential for pathogenicity, whereas deletion of four clusters (6A, 10A, 19A, and 5B) reduces or abolishes pathogenicity and deletion of cluster 2A results in increased pathogenicity (Table 1). Microscopic examination of infected plants indicates that cluster 5B null mutants cause arrest early during penetration of the epidermis, and cluster 19A deletion mutants penetrate the host cell and grow inside the plant tissue but fail to induce large tumors and do not produce teliospores (Kämper

et al., 2006). These observations indicate that these clusters play roles at different steps of the infectious cycle. Deletion analysis of individual genes in the clusters required for pathogenicity will likely provide insights about their specific role during infection. A key question is to determine if these predicted secreted proteins are translocated inside the plant cell, where they might interfere with growth control or attenuate host defense responses, or whether they exert their effect by interaction with host receptors.

mrb1, a Link to Mitochondria

mrb1 codes for a putative protein of 274 amino acids, of the p32 family of proteins, that localizes to the mitochondrion (Bortfeld et al., 2004). *mrb1* null mutants do not affect growth of yeast-like cells or the formation of dikaryotic filaments on charcoal agar. Inoculation of plants with *a1 b1 Δmrb1* + *a2 b2 Δmrb1* strains results in a dramatically reduced tumor response (5% for the mutant versus 92% for the wild type). Microscopic examination of infected plants indicates that few hyphae are present and these are mostly unbranched. Thus, *mrb1* is required for hyphal branching and proliferation (Bortfeld et al., 2004).

The Developmental Program Leading to Teliospore Formation

The developmental program leading to teliospore formation is essential for completion of the fungal life cycle.

Hyphal Fragmentation and the *fuz1* Gene

The *fuz1* gene codes for a MYND zinc finger domain protein of 1,421 amino acids (Chew et al., 2008). The MYND domain is a highly conserved zinc finger domain in all eukaryotes and is proposed to mediate protein-protein interactions, in particular, with regulatory proteins (see Chew et al., 2008, and references therein). *fuz1* is required for cell morphogenesis, cell separation, cell wall integrity, and inhibition of pigment production. On charcoal media, *fuz1* mutants are unable to form filaments, yet they are able to form filaments in planta and to proliferate inside plant cells. Fuz1 mutants reduce the tumor response: the tumors are small and restricted in location (Table 1). Because fungal proliferation in the plant is not altered in *fuz1* mutants, the reduced tumor response and restricted location of tumors likely reflect a direct function of *fuz1* in the ability to invade host tissues and induce tumors (Banuett and Herskowitz, 1996). It is possible that the MYND domain of Fuz1 interacts with a regulator of tumor induction. The tumors formed are devoid of teliospores, and this is due to a specific arrest at the hyphal fragmentation stage during teliospore formation (Banuett and Herskowitz, 1996). *fuz1* mutant hyphae are unable to fragment; they stretch abnormally within plant cells and in many cases rupture, forming unusual cytoplasmic projections indicative of a weak cell wall. Fungal growth ceases, and the tumors are white, due to the absence of teliospores (Banuett and Herskowitz, 1996). The defect in hyphal fragmentation together with the observation that *fuz1* mutants confer a cell separation defect in yeast-like cells in culture indicates that Fuz1 is required at the junction of cells in both cell types. Fuz1 may recruit components necessary for cell wall-remodeling events at the junction of cells via its MYND domain (Chew et al., 2008).

A Histone Deacetylase

The *hda1* gene was identified in a screen to isolate mutants that express the *egl1* gene in a *b*-independent manner (*egl1* is normally expressed in a *b*-dependent manner) (Reichmann et al., 2002). *hda1* codes for a protein of 570 amino acids with similarity to histone deacetylases. In *Δhda1* mutants that lack an active b protein, expression of several *b*-regulated genes (*egl1*, *dik1*, *hum2*, and *lga2*) is increased. If the strains contain an active b protein and the *Δhda1* mutation, expression of these genes is even higher than in strains with wild-type *hda1*. Thus, Hda1 appears to negatively regulate expression of these genes independently of the *b* locus. Expression of the *b* genes (*bW* and *bE*) is also increased.

In addition to the above genes, *ssp1* and *mig1* gene expression is increased in *Δhda1* strains (see "Genes in Search of a Function in Pathogenicity" below). Inoculation of plants with *hda1* null mutants results in formation of tumors that lack teliospores (Table 1) (Reichmann et al., 2002). Karyogamy is not altered. The *Δhda1* mutation appears to block cell rounding. These observations indicate that *hda1* is required for teliospore formation and may act at the cell rounding stage or prior to it (Reichmann et al., 2002). The block is distinct from that observed with *fuz1* mutants.

A Putative Partner of Hda1: Rum1

The *rum1* gene was identified using a screen similar to that used for identification of the *hda1* gene. The Rum1 (regulator *U. maydis*) protein (2,289 amino acids) shows similarity to human protein Rbp2 (retinoblastoma binding protein 2) (Quadbeck-Seeger et al., 2000). It contains an ARID domain (a DNA-binding motif), two PHD-finger domains (zinc binding domains found in nuclear proteins thought to mediate protein-protein interactions), a PLU domain (a conserved domain of unknown function), and a bipartite nuclear localization signal (Quadbeck-Seeger et al., 2000). Like Hda1, Rum1 is involved in expression of several *b*-regulated genes.

rum1 null mutants exhibit no phenotype in culture. Inoculation of plants with *Δrum1* strains results in delayed tumor formation, and the tumors are devoid of teliospores (Table 1). Teliospore development appears to be arrested at the cell-rounding stage, similar to that of *hda1* mutants; however, as for *hda1*, it is not clear whether the step altered in the *rum1* mutants is cell rounding or an earlier step. Karyogamy is not altered (Quadbeck-Seeger et al., 2000). Given the similar phenotypes of *hda1* and *rum1* mutants, the two genes are likely to act in the same pathway required for teliospore formation and have been proposed to form a complex that negatively regulates genes also regulated by *b* (Quadbeck-Seeger et al., 2000).

A Putative Target of PKA: Hgl1

The *hgl1* gene was identified as a suppressor of the filamentous phenotype conferred by deletion of *adr1* (Dürrenberger et al., 2001). The *hgl1* gene codes for a protein of 612 amino acids with no similarities in the databases. It contains eight potential PKA phosphorylation sites (RRXS). In vitro-synthesized Hgl1 protein is phosphorylated by partially purified *U. maydis* PKA (Dürrenberger et al., 2001). Whether this phosphorylation is important for the in vivo function of Hgl1 remains to be determined. The *hgl1* gene is expressed at a low level in haploid cells and is induced upon mating and filament formation. *Δhgl1* mutants form colonies that are darkly pigmented, similar to *fuz1* mutants, and reduce filament formation on charcoal agar. Inoculation of plants with *hgl1* null mutants results in the formation of tumors that are larger than those induced by wild-type strains and are devoid of teliospores (Table 1). Microscopic examination of infected plants revealed that the *Δhgl1* mutant

arrests development after hyphal fragmentation (Dürrenberger et al., 2001). The *Δhgl1* mutation does not alter karyogamy. A model was proposed in which Adr1 negatively regulates Hgl1, which in turn positively regulates filamentous growth and teliospore formation and negatively regulates pigment production and budding growth (Fig. 5) (Dürrenberger et al., 2001).

Components of the cAMP Pathway: *gpa3* and *ubc1*

An activated allele of *gpa3*, $gpa3^{Q206L}$, confers a glossy colony morphology and an elongated cell morphology phenotype (Regenfelder et al., 1997). This mutation does not alter formation of dikaryotic filaments on charcoal agar. $gpa3^{Q206L}$ strains of opposite mating type induce tumor formation, in contrast to the absence of tumors in infections with *gpa3* null mutants. The $gpa3^{Q206L}$-induced tumors lack teliospores (Table 1). Microscopic observation of fungal development within the tumors indicates that the mutant likely arrests after the hyphal fragmentation stage and is unable to undergo cell rounding (Krüger et al., 2000). These studies and those described above ("cAMP and Early Steps in Infection") demonstrate that *gpa3* has multiple roles during the infectious cycle.

A specific allele of *ubc1* ($ubc1^{R321Q}$), identified in a screen for *ubc1* mutants that exhibit a colony morphology phenotype similar to that of $gpa3^{Q206L}$, leads to a defect in teliospore production and attenuated tumor response. Tumor induction was found to be intermediate between that of infections with wild-type and $gpa3^{Q206L}$ strains. The $ubc1^{R321Q}$ mutation leads to arrest of teliospore development at a step close to that observed with $gpa3^{Q206L}$ (Krüger et al., 2000).

Taken together, the above observations and those described above in "cAMP and Early Steps in Infection" indicate that *gpa3* and *ubc1* have multiple roles during the infectious cycle and that modulation of cAMP levels is critical for symptom development and fungal differentiation in the plant (Krüger et al., 2000).

Septins and Teliospore Cell Wall: *sep3*

The *sep3* gene was identified using SAGE (see "Phosphate Acquisition: *vtc4* and a Link to cAMP" above) (Larraya et al., 2005). *sep3* codes for a homologue of *S. cerevisiae CDC11*, a septin involved in cytokinesis and cell morphogenesis. The *sep3* gene is required for normal cell morphogenesis and septation: *sep3* null mutants form cell clumps, and their cells are wider than those of the wild type, have a cell separation defect, and form a septum in the middle. The compartments can be multinucleate (Boyce et al., 2005). Formation of conjugation tubes or filaments on charcoal agar is not altered. The *sep3* gene is required for full symptom development: inoculation of plants with *Δsep3* null mutants of opposite mating type results in reduced symptom development. The tumors appear to have increased numbers of immature teliospores. The cell wall of mature spores was not as thick as that of teliospores formed by wild-type strains (Table 1) (Boyce et al., 2005). These observations indicate that *sep3* is required for normal maturation of teliospores, specifically in formation of the specialized cell wall. A precedent for a role of septins in cell wall formation is provided by studies in *S. cerevisiae*, wherein septins were shown to be required for prospore wall formation during sporulation (see, for example, Fares et al., 1996). *sep3* null mutants produce teliospores that germinate and form aberrantly shaped promycelia.

A link between *sep3* and the cAMP pathway is supported by the observation that *sep3* null mutants suppress the *ubc1* cell morphology phenotype of multiple budding and the cell separation defect (Boyce et al., 2005). It would be interesting to determine if *sep3* suppresses the *ubc1* defect in planta (*ubc1* mutants proliferate in the plant but do not form tumors). Sep3 is also required for lipid-induced filamentation, a response that requires the cAMP pathway: *Δsep3* mutants are unable to filament when exposed to a variety of lipids (Boyce et al., 2005).

Endocytosis: a tSNARE

yup1 codes for a tSNARE (target membrane-associated soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor) that localizes to early endosomes (Fuchs et al., 2006). Inoculations of plants with $yup1^{ts}$ strains of opposite mating type results in a full tumor response at the permissive temperature (22°C), whereas tumor induction was abolished at the restrictive temperature (34°C) (Table 1). Because $yup1^{ts}$ mutants fail to respond to pheromones, it is likely that the effect on tumor formation is due to failure to form the filamentous dikaryon in planta, though this was not determined. If plants are inoculated with $yup1^{ts}$ strains at the permissive temperature and after 3 days are shifted to the restrictive temperature, the dikaryotic hyphae that form are wider and irregularly shaped compared to wild-type hyphae. These mutant hyphae are able to penetrate and induce tumors. The tumors are devoid of teliospores (Table 1). Because hyphal fragments are present in the tumors, *yup1* appears to be required in a step after hyphal fragmentation (Fuchs et al., 2006). Teliospores obtained from plants infected with $yup1^{ts}$ at the permissive temperature germinate normally at the permissive temperature, whereas at the restrictive temperature, germination is reduced. The teliospores that are able to germinate exhibit a morphologically altered promycelium. This aberrant promycelium can still produce basidiospores (Fuchs et al., 2006). The authors conclude that *yup1* is necessary for teliospore formation and germination and for morphology of the promycelium. Taken together, the above observations support a role of endocytosis at different steps in the infectious cycle.

Teliospore Germination, Meiosis, and Generation of the Haploid Phase

Differential Gene Expression during Teliospore Germination

The first large-scale analysis of changes in gene expression during teliospore germination was carried out by Zahiri et al. (2005) using microarrays of a set of 3,918 nonredundant cDNAs. RNA isolated from dormant (reference RNA) and germinated teliospores 4 and 11 h after induction of germination (experimental RNA) were hybridized to the cDNA microarray to identify genes that were differentially expressed. The downregulated genes at the 4-h time point fell into several functional categories: chromosome structure, recombination and DNA repair, transcription, translation initiation, protein turnover, iron-sulfur cluster assembly, stress response, energy metabolism and metabolic transport, extracellular degradation, and mitochondrial biogenesis (Zahiri et al., 2005). The upregulated genes at the 4-h time point included *rad13* (DNA repair), genes coding for cell signaling components and protein synthesis, and *U. maydis*-specific genes coding for hypothetical proteins. The genes upregulated at 11 h include genes involved in DNA repair, translation, post-translational modification, protein assembly, cell wall synthesis (chitin synthase [chs2]), metabolism, transport, and mitochondrial biogenesis. The genes upregulated at 11 h were not upregulated at 4 h postgermination (Zahiri et al., 2005).

BRCA2 and Rad51 Homologues Link Recombination to Teliospore Germination

Interestingly, two of the genes upregulated during teliospore germination are *brh2* and *rad51* (Zahiri et al., 2005). *brh2* and *rad51*, in addition to their role in recombination, are essential for teliospore germination: *brh2* or *rad51* null mutants are unable to produce a promycelium (Table 1) (Kojic et al., 2002). The Brh2 protein is a homologue of mammalian BRCA2; BRCA2 homologues are not present in the genomes of *S. cerevisiae* or *S. pombe* (Kojic et al., 2002). *rad51* codes for a Rad51 homologue (Kojic et al., 2002). Interestingly, *rec2* null mutants germinate and produce a promycelium, but meiosis aborts, and the promycelium does not form basidiospores (Table 1) (Kojic et al., 2001). *rec2* codes for a divergent Rad51 paralog involved in DNA recombination (Kojic et al., 2001). These observations support the notion that teliospore germination and completion of meiosis are coordinately regulated.

The *fuz2* Gene

The uncharacterized *fuz2* gene (Banuett, 1991) is required for teliospore germination. *fuz2* is required for filament formation on charcoal agar, but it is not required for tumor induction or teliospore production. Its role is specific for teliospore germination (Table 1).

Cell Cycle: a Pcl1,2 Cyclin

pcl12 is one of seven genes in the *U. maydis* genome that code for predicted cyclins with similarities to the *S. cerevisiae* Pcl cyclins of the Pho80 and Pcl1,2 subfamilies (Flor-Parra et al., 2007). In *S. cerevisiae*, these cyclins associate with Pho85 and regulate various cellular processes. The *U. maydis* orthologue of Pho85 is Cdk5 (Castillo-Lluva et al., 2007) and has been shown to associate with Pcl12 (Flor-Parra et al., 2007). Loss of *pcl12* leads to altered morphology of the yeast-like cell, blocks the switch to *b*-dependent filamentous growth, and causes disorganization of actin patches. Overexpression of *pcl12* induces filament formation in a Cdk5-dependent manner, and the nucleus in these filaments is arrested in the G2 phase. Inoculation of plants with *pcl12* null mutants does not affect tumor formation or teliospore production, although the hyphae exhibit aberrant morphology. Teliospore germination is blocked; in rare instances short aberrant promycelia are formed (Table 1) (Flor-Parra et al., 2007).

OTHER GENES REQUIRED FOR PATHOGENICITY

The genes described below are required for symptom development, but the specific step at which they are required has not been determined. This is also true for some of the genes described in the previous section (Table 1).

smu1

smu1 codes for a PAK. In addition to the kinase catalytic domain, it contains a Cdc42-Rac interactive-binding (CRIB) domain. A *smu1* null mutation results in delayed formation of filaments on charcoal agar. *smu1* is also required for a full tumor response (Table 1). The presence of only chlorosis suggests an early defect in the infectious process (Smith et al., 2004).

ras2

The *ras2* gene was identified as a suppressor of the filamentous phenotype of an *adr1* mutant (Lee and Kronstad, 2002). *ras2* codes for a protein of 192 amino acids with similarity to RAS2 of *S. cerevisiae*. Deletion of *U. maydis ras2* results in aberrant morphology and loss of polarity: cells are rounder and wider. A haploid strain carrying an activated *ras2* allele ($ras2^{V116}$) exhibits filamentous growth. This phenotype requires at least two functional components of the pheromone response pathway, Fuz7/Ubc5 and Ubc3/Kpp2 (Fig. 2), but it does not require an intact Prf1, the pheromone response transcriptional activator (Lee and Kronstad, 2002). These results suggest that Ras2 acts upstream of the MAPK cascade and that the output of the *ras2* signal may involve another transcriptional activator. *ras2* is required for pathogenicity. *ras2* null mutants do not form conjugation tubes or filaments in culture, which may also be true in planta and would explain the lack of pathogenicity of *ras2* null mutants (Lee and Kronstad, 2002). The activated *ras2* allele, ras^{Val116}, is hypervirulent, but details of the phenotype were not provided (Table 1) (Lee and Kronstad, 2002).

myp1

The *myp1* gene was identified as a mutation that suppresses the filamentous phenotype of a haploid engineered to carry different *a* and *b* alleles (Giasson and Kronstad, 1995). The predicted protein of 1,150 amino acids is rich in Ser, Ala, and Pro and has no similarities to proteins in the databases. *myp* mutants attenuate filament formation on charcoal agar. Inoculation of plants with *myp1* mutants results in reduced tumor response (Table 1) (Giasson and Kronstad, 1995).

glo1

The *glo1* gene codes for a predicted protein with similarity to a glyoxal oxidase from *P. chrysosporium*. Glyoxal oxidases are not present in the yeasts. *glo1* is necessary for filament formation on charcoal agar and for pathogenicity (Table 1). The Glo1 protein appears to localize to the membrane, oxidizes a series of small aldehydes, and produces H_2O_2 (Leuthner et al., 2005).

RNA Binding Proteins and Pathogenicity: Khd4 and Rrm4

Khd4 and Rrm4 were identified in a search of the *U. maydis* genome for proteins with signature RNA binding motifs (PUM, KHD, DSRM, and RRM) (Becht et al., 2005). *khd4* codes for a predicted protein containing a K homology domain (KHD) (Becht et al., 2005). Deletion of *khd4* reduces filament formation on charcoal agar, due to defects in conjugation tube formation as well as filamentous growth per se. Inoculation of plants with *Δkhd4* strains of opposite mating type results in an attenuated tumor response (Table 1). *rrm4* codes for a predicted protein containing an RNA recognition motif (RRM). Deletion of *rrm4* reduces filament formation on charcoal agar, and the hyphae produced are shorter than those from the wild type. The defect in filament formation was shown to be independent of cell fusion (Becht et al., 2005). Rrm4 is required for pathogenicity.

GENES IN SEARCH OF A FUNCTION IN PATHOGENICITY: *ssp1* AND THE *mig* AND *pig* CLUSTERS

The *mig* Genes

The *mig* genes (for maize induced gene) were the first genes identified that are induced during the biotrophic phase. *mig1* is not linked to a cluster of five highly similar *mig*-related genes (*mig2-1*, *2-2*, *2-3*, *2-4*, and *2-5*), which are arranged in tandem as direct repeats in a 7.1-kb chromosomal region

(Basse et al., 2002). The *mig* genes code for small secreted cysteine-containing hydrophilic proteins with no matches in the databases. The predicted proteins contain a signal sequence with similarity to that of Egl1, a secreted endoglucanase (Basse et al., 2002). The *mig2* gene cluster and the *mig1* gene are not expressed in haploids but are expressed in diploids heterozygous at *a* and *b*. The regulatory subunit (Ubc1) of cAMP-dependent protein kinase is required for expression of *mig2-1*. This observation suggests that the *mig* genes are subject to regulation by the mating-type loci and by cAMP.

The *mig1* gene is upregulated in the plant beginning 2 days after inoculation, and expression peaks 6 dpi (approximately 1,000-fold greater than expression in the *a* and *b* heterozygous diploid) and declines thereafter (Basse et al., 2000). Expression of GFP under control of the *mig1* promoter (*Pmig1*::GFP) was not detected in filaments and appressoria. After penetration, hyphae exhibited weak fluorescence, which increased 2 days later and remained high during subsequent proliferation and tumor formation. It was not detected in the teliospores (Basse et al., 2000). The *mig2-1* and the other genes in the cluster are upregulated in the plant beginning 2 days after inoculation, with a peak around 4 to 6 dpi (Basse et al., 2002). A *mig2-1*::GFP fusion protein is expressed after penetration and throughout proliferation, but expression decreases after hyphal fragmentation and is absent from teliospores (Basse et al., 2002). Deletion of *mig1* or the *mig2* gene cluster has no effect on pathogenicity.

The *mzr1* Gene: a Regulator of the *mig* Genes Also Regulates Many Other Genes

The *mig2-5* promoter contains nine repeats of the triplet CCA, five of which match the consensus CCAC/AC/A and are critical for induction in the plant (see Zheng et al., 2008, and references therein). Precedence exists for Myb-type or C_2H_2 regulatory proteins recognizing such sequences. A search of the *U. maydis* genome sequence identified at least 39 genes (*znf*) coding for the predicted C_2H_2 zinc finger domain proteins (Zheng et al., 2008). Deletion analysis of individual *znf* genes revealed that only *zfn22* null mutants reduced expression of a *Pmig2-5*::eGFP reporter construct in the plants. The gene was renamed *mzr1* (*mig* zinc finger regulator). It codes for a protein of 1,614 amino acids that localizes to the nucleus. Deletion of *mzr1* has no effect on pathogenicity (Zheng et al., 2008).

Results from mRNA profiling analysis of a strain expressing wild-type levels of *mzr1* versus a strain overexpressing *mzr1* fourfold indicate that several of the upregulated genes belong to some of the gene clusters coding for putative secreted proteins (see "Gene Clusters for Putative Secreted Proteins: Effectors of Tumor Induction or Antagonists of Plant Defense?" above) uncovered in the analysis of the genome sequence (Kämper et al., 2006). Most of these genes are induced in planta, and their expression peaks 2 dpi, similar to the pattern observed for *mig2-5*. *mzr1* itself is upregulated 30-fold in planta, consistent with a role in gene induction during biotrophic growth. The induced expression of *mzr1* in planta and its role in regulating genes other than the *mig* genes vis-à-vis its dispensability for pathogenicity are rather surprising.

The *pig* Genes

Plant-induced genes (*pig* genes) were identified using a combination of REMI and enhancer trapping (Aichinger et al., 2003). Of 2,350 insertion mutants that were analyzed, only 3 were shown to express GFP upon contact with the plant. One of these insertions occurred in the *pig2* gene that codes for a putative protein of 550 amino acids with similarity to protein disulfide isomerases. The gene is induced on charcoal agar, and this expression is drastically increased in tumor tissue. Another insertion occurred in the *p* locus, which contains 11 genes in a 24-kb DNA segment. Five of the 11 genes (*pig3*, *pig4*, *pig5*, *pig6*, and *pig7*) are upregulated in the plant at different stages in the infectious cycle (see Aichinger et al., 2003, for details). Deletion of *pig2* or of the *p*-locus has no effect on pathogenicity (Aichinger et al., 2003).

The *ssp1* Gene

Ssp1 (sporulation specific protein) was identified as a protein expressed exclusively in teliospores. The *ssp1* gene codes for a protein of 1,064 amino acids with similarity to linoleate diol synthase (Huber et al., 2002). Deletion of *ssp1* has no effect on cell morphology, filamentous growth, pathogenicity, teliospore production, or teliospore germination. Expression of *ssp1* is induced in the teliospore. An Ssp1::GFP protein fusion localizes to mature teliospores and to germinating teliospores, where it localizes to lipid bodies. The presence of lipid bodies was not altered by the *Δssp1* mutation.

Although the approach of gene identification based on gene expression during biotrophic growth has provided information about genes expressed at distinct phases of the infectious cycle, it has failed to identify key players of the infectious cycle. One positive outcome of these studies is the identification of genes whose promoters may prove useful in the regulation of other genes in a stage-specific manner.

PLANT DEFENSE AND FUNGAL COUNTERDEFENSE

Plant defense against pathogen invasion occurs by a variety of mechanisms, one of which involves the production of reactive oxygen species (ROS) by membrane-localized NADPH oxidases. Peroxidases bound to the cell wall use the generated H_2O_2 or phenolic substrates in the synthesis of lignin and other phenolic compounds that protect against invasion. In turn, pathogens use a variety of mechanisms to counter the defense mechanisms of plants, one of which involves detoxification of ROS.

yap1

U. maydis yap1 was identified in a search of the genome for homologues of *S. cerevisiae YAP1* (Molina and Kahmann, 2007). Budding yeast *YAP1* codes for a basic leucine zipper transcription factor required for tolerance to oxidative stress. The gene is activated by H_2O_2. Growth of *U. maydis yap1* null mutants is inhibited by H_2O_2. Subcellular localization of a Yap1::eGFP(3×) fusion protein is modulated by H_2O_2: in its presence, the fusion protein localizes to the nucleus, while in its absence, the fusion protein is present throughout the cell. Deletion of *yap1* results in a reduced tumor response, but microscopic observation of the infectious process was not reported (Table 1). Because the tumor response was not abolished, the authors concluded that another protein is able to detoxify ROS or that there is no defense response once penetration occurs and that the role of Yap1 is to prevent ROS accumulation early in infection (Molina and Kahmann, 2007). The *yap1* gene is induced during early stages of biotrophic growth.

Transcriptional profiling analysis of *yap1* versus *yap1* null strains in the presence or absence of 5 mM H_2O_2

revealed that Yap1 downregulates 221 genes and upregulates 203 others (Molina and Kahmann, 2007). The Yap1-regulated genes code for putative proteins that fall into three categories: (i) ROS-detoxifying enzymes (peroxidases and catalases); (ii) proteins involved in biosynthesis of low-molecular-mass antioxidants (ascorbic acid, glutathione, tocopherols, NADH, and NADPH); and (iii) enzymes that regenerate the reduced form of antioxidants (Molina and Kahmann, 2007). Two genes that code for putative peroxidases, heme peroxidase (*um10672*) and cytochrome *c* peroxidase precursor (*um01947*), might be involved in detoxification of ROS (see Molina and Kahmann, 2007, for a list of genes induced by Yap1). A *um10672* (heme peroxidase) null mutant is more sensitive to H_2O_2 than the *Δyap1* mutant and ROS accumulation is not blocked, whereas in a *um01947* (cytochrome *c* peroxidase precursor) mutant, ROS accumulation is blocked. The authors concluded that *um10672* is likely the peroxidase that degrades ROS during early stages of infection. Both mutants reduced the tumor response (Table 1) (Molina and Kahmann, 2007). The fact that mutation in either of these genes or *yap1* reduces but does not abolish tumor induction suggests that the fungus uses multiple activities to cope with plant defense or that there is no defense response after penetration.

Plant Genes Upregulated during *U. maydis* Infection

Recent analysis has turned attention to expression profiling of the plant transcriptome in response to infection by *U. maydis* (Basse, 2005; Doehlemann et al., 2008b). These studies indicate that *U. maydis* elicits a plant defense response early in infection, which is attenuated at later stages. The fungus also elicits differential expression of plant genes involved in secondary metabolism, antioxidants, hormone signaling, and photosynthesis (Doehlemann et al., 2008a).

CONCLUDING REMARKS

This chapter provides an overview of steps involved in the interaction of *U. maydis* with its host maize and a brief summary of genes known to play a role in this process. Some of these genes are required at multiple steps, whereas others appear to act at discrete times. Analysis of mutants in combination with sophisticated imaging techniques and the application of expression profiling of individual infected versus noninfected cells will allow a more precise dissection of the infectious process and identification of the genes that are differentially regulated in the partners of this "apparent" harmonious interaction. Much remains to be elucidated about the *U. maydis*-maize interaction. For example, does the fungus translocate proteins that attenuate the defense response or induce tumor formation? What is the nature of the plant signals that induce fungal differentiation?

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Necrotrophic Fungi: Live and Let Die

MARTY DICKMAN, JAN VAN KAN, AND CHRISTOPHER LAWRENCE

THE RELEVANCE OF NECROTROPHIC PLANT PATHOGENIC FUNGI

Necrotrophic fungi are pathogens that obtain nutrients from dead cells. As a result, this group of fungi inflict substantial tissue damage on their hosts in advance of, and during, colonization. Although they represent just 4% of fungal diversity, they can cause up to 80% in yield losses and thus are of significant economic importance. These fungi are generally aggressive pathogens and in some cases possess extremely broad host ranges. Our understanding and identification of key determinants required for necrotrophic disease are not well elucidated. Traditionally, necrotrophic fungi have been viewed as relatively indiscriminate, causing disease by sheer force and aggression. Consistent with this premise, their offensive weaponry includes nonspecific toxins and numerous cell wall-degrading enzymes; thus, the fungus simply overwhelms the "passive" host plant who has little to say in the matter or little influence on the outcome. Of considerable interest and relevance to this chapter is the accumulating evidence suggesting that plant-necrotroph relationships have been oversimplified and in fact involve a complex exchange between both partners that is necessary before resistance or disease is observed.

Necrotrophs have been studied since the days of de Bary in the 1880s. The bulk of studies with these pathogens have focused on disease control, spray regimes, management strategies, inoculation procedures, bioassays, and description of the infection processes. Mechanistic analyses of necrotrophs were limited by the relative lack of experimental tractability. This has now changed, and tools have been (and are continuing to be) developed for pathogen manipulation, resulting in considerable increases in experimental power. In addition, the remarkable progress in fungal genomics and genome sequencing efforts have significantly catalyzed advances in understanding all fungal pathogen groups. As a result, our understanding of necrotrophic fungal biology, development, and pathogenicity is rapidly expanding.

In contrast to necrotrophs, biotrophic pathogens (e.g., rusts, smuts, and mildews) require living tissue in order to obtain nutrients, grow, and reproduce. Several biotrophs cannot be cultured axenically. Hemibiotrophs exhibit features from both of these lifestyle classification schemes, and the regulation of lifestyle transitions is poorly understood. The biotrophic fungi often exhibit gene-for-gene-type interactions (Ellis et al., 2007), and such systems, particularly from the plant side, have received considerable attention in the more fundamental studies of fungus-plant interactions. In the past decade, the regulatory mechanisms underlying infection-related morphogenesis and pathogenicity have been identified and analyzed, with the bulk of such studies performed with biotrophic (*Ustilago* spp.) and hemibiotrophic (*Magnaporthe grisea* and *Colletotrichum* spp.) pathogens.

In this chapter three major fungal necrotrophs, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Alternaria brassicicola*, are compared and contrasted. The group of necrotrophic fungi that are renowned for production of host-specific toxins, such as *Cochliobolus* spp., *Pyrenophora tritici-repentis*, *Stagonospora nodorum*, are not discussed. All three fungi discussed in this chapter have recently completed genome sequences (Table 1). Given the variety in pathogenicity-related mechanisms involved (some of which appear to be indiscriminate of host), accumulating evidence supports the fact that necrotrophic pathogens interact with their hosts in a manner much more subtle than originally considered and that signaling between the necrotroph and the host plays a significant role in the lifestyle of these pathogens (van Kan, 2006).

S. SCLEROTIORUM

S. sclerotiorum has arguably one of the broadest host ranges known for a fungal plant pathogen (over 400 plant species), mainly dicots (Boland and Hall, 1994; Purdy, 1979; Tu,

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TABLE 1 Features of the genome sequences of *S. sclerotiorum*, *B. cinerea*, and *A. brassicicola*

Feature	*S. sclerotiorum*[a]	*B. cinerea* B05.10[b]	*B. cinerea* T4[c]	*A. brassicicola* ATCC 96836[d]
Sequencing method	Genomic libraries (4, 10, and 40 kb)	Genomic libraries (4, 10, and 40 kb)	Genomic libraries (3 and 10 kb), BAC[e] ends	Random shotgun, BAC and fosmid ends
Genome sequence length (Mb)	38.3	42.3	39.5	31
Optical map available?	Yes	No	No	No
No. of chromosomes	16	16 (estimated)	16 (estimated)	9
Coverage	8×	4.6×	10×	6.4×
No. of contigs	679	4,534	2,281	4,039
No. of scaffolds	36	588	118	838
N50 length (Mb)	1.6	0.26	0.56	2.4
Sequence quality	97.8% > Q40	89% > Q40	98.8% > Q40	98.8% > Q20
No. of gene models	12,031	13,806	12,796	10,688
No. of ESTs[f] available	>70,000	>90,000	6,430	

[a]http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/.
[b]http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/.
[c]http://urgi.versailles.inra.fr/projects/Botrytis/.
[d]http://www.genome.wustl.edu/genome.cgi?GENOME=Alternaria%20brassicicola.
[e]BAC, adenylate cyclase.
[f]EST, expressed sequence tag.

1997). Diseases caused in economically important plants by *S. sclerotiorum* occur worldwide (Steadman, 1979), cause considerable damage, and have proven difficult to control; and host resistance to this fungus has been inadequate. Annual losses of crops from diseases caused by *S. sclerotiorum* are in the multimillion dollar range (http://www.whitemoldresearch.com).

Despite the agroeconomic importance of diseases caused by *S. sclerotiorum*, mechanistic studies related to pathogenic development have been few. One reason for the limited molecular investigations has been the perceived complexity, i.e., the quantitative nature of pathogenicity and disease resistance. Molecular tool development has also been hampered by the multinucleate nature of *Sclerotinia* hyphae and the complete lack of conidial production. Many of these technological hurdles have now been overcome, and *S. sclerotiorum* is now a tractable system for molecular genetic studies. Stable transformation, green fluorescent protein (GFP) expression (Lorang et al., 2001), restriction enzyme-mediated integration mutagenesis, antisense inhibition of gene expression (Chen et al., 2004), RNA interference (Erental et al., 2007), and functional gene analysis via gene replacement (Rollins, 2003) are now in place.

Sclerotia

Sclerotia are a primary determinant contributing to the pathogenic success of *S. sclerotiorum*. The sclerotium of *S. sclerotiorum* is a multicellular, highly pigmented (melanized), rigid, asexual, resting/overwintering structure composed of condensed vegetative hyphal cells that become interwoven and aggregate together, and it is capable of surviving years in soil (Chet and Henis, 1975; Adams and Ayers, 1979; Tourneau, 1979; Willetts and Bullock, 1992; Erental et al., 2008). Sclerotia germinate to produce saprophytic or infectious mycelia, or they germinate carpogenically to form apothecia. The importance of sclerotia for the pathogenic success of this fungus is underscored by the fact that sclerotia are the primary survival structures of this fungus upon which all other developmental phases of the fungus depend (Fig. 1).

Oxalate

Effective pathogenesis by *S. sclerotiorum* requires the secretion of oxalic acid (OA). The role of OA in fungal pathogenicity was originally demonstrated using a genetic approach. Prototrophic mutants were obtained by UV irradiation of ascospores. Survivors of this mutagenic treatment were plated on media containing the high-pH indicator dye bromophenol blue. Deficiency in OA production was determined from isolates that could no longer acidify the media via OA synthesis and thus were unable to change the color of the indicator dye. Gas chromatography and high-pressure liquid chromatography confirmed that selected mutants did not produce OA, although they grew in a fashion similar to that of the wild type. A consistent feature of all of the OA^- mutants was the inability to produce sclerotia. A revertant to OA expression also reverted to sclerotial development. A link between secondary metabolism and development was suggested (Godoy et al., 1990), but despite considerable effort, this potential linkage and the gene(s) responsible remain to be identified. Importantly, when OA-defective mutants were inoculated into bean pods, all of the mutants were nonpathogenic. This has been subsequently shown to be the case for several dicotyledonous host plants. Thus, OA was demonstrated to be a pathogenicity determinant in *S. sclerotiorum* and possibly linked with sclerotial development. Given that OA^- mutants were nonpathogenic, possible modes of action for this organic acid were explored. Oxalate secretion might enhance *S. sclerotiorum* virulence in at least four ways (reviewed by Dutton and Evans, 1996). First, because several of the fungal enzymes secreted during invasion of plant tissues (e.g., polygalacturonases) have maximal activities at low pH, various researchers have postulated that OA might aid virulence by shifting the apoplastic pH to a value more suited for enzymatic degradation of plant cell walls (Bateman and Beer, 1965). Second, OA may be

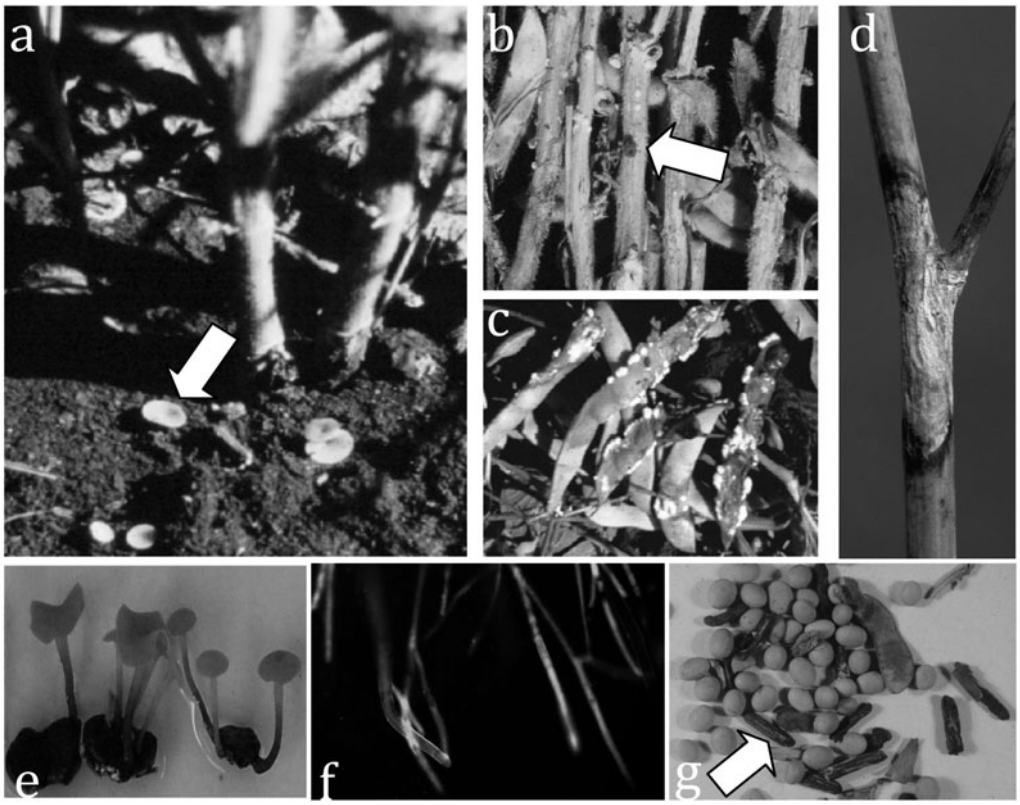

FIGURE 1 Typical symptoms caused by *S. sclerotiorum* and structures of the fungus. (a) Apothecia (arrow) below a soybean canopy. (b) White mold on dry bean stalks with sclerotia (arrow). (c) Symptoms of white mold on dry bean: water-soaked lesions with fluffy mycelium. (d) Oilseed rape infected with *S. sclerotiorum* showing bleached stem tissue and necrotic side branch. (e) Carpogenic germination of sclerotia resulting in apothecia with ascospores. (f) GFP-tagged *S. sclerotiorum* under fluorescence microscopy (De Silva et al., 2005). (g) Sclerotia in a soybean harvest sample (arrow).

directly toxic to host plants because of its acidity, and the secretion of OA has been suggested to weaken the plant, thereby facilitating invasion (Noyes and Hancock, 1981). Oxalate crystals are known to form in *S. sclerotiorum*-infected plants and, when decompartmentalized, can result in vascular plugging. Chelation of cell wall Ca^{2+} by the oxalate anion has been proposed both to compromise the function of Ca^{2+}-dependent defense responses and to weaken the plant cell wall (Bateman and Beer, 1965). Recently, OA has been reported to interfere with the function of guard cells, resulting in abnormal stomatal opening and inhibition of abscisic acid-induced stomatal closure during infection (Guimarães and Stotz, 2004). Thus, it is evident that this "simple" dicarboxylic acid is used by the fungus in a myriad of ways when interacting with host plants. The importance of OA for successful infection by *S. sclerotiorum* is illustrated by the fact that OA mutants are nonpathogenic, even though the fungus maintains its full arsenal of cell wall-degrading enzymes (Godoy et al., 1990). Although each of these hypotheses has logical appeal, evidence supporting them as primary determinants of pathogenic success is incomplete, and arguments against their validity have also been made (Dutton and Evans, 1996). As described below, additional activities have emerged as being crucial for the effectiveness of this multifunctional organic acid.

S. sclerotiorum acidifies its ambient environment by producing OA. Sclerotial development is favored by acidic ambient pH conditions but inhibited by neutral ambient pH; thus, OA production and sclerotial development are inversely related (Rollins and Dickman, 1998). Transcripts encoding the endopolygalacturonase gene *pg1* accumulate maximally under acidic culture conditions. OA synthesis is greatest at neutral pH; as OA levels increase, the pH is lowered, possibly serving as a signal for sclerotial development. In *Aspergillus nidulans*, many gene products with pH-sensitive activities have been shown to be regulated by a common transcription factor, *Pac*C (Caddick et al., 1986). The putative *S. sclerotiorum* homolog (*Pac1*) was cloned by PCR. The three zinc finger domains of the Pac1 protein are similar in sequence and organization to the zinc finger domains of the *A. nidulans* PacC. The promoter of *Pac1* contains eight PacC consensus binding sites, suggesting that this gene, like its homologs, is autoregulated. In accordance, the accumulation of *Pac1* transcript paralleled increases in ambient pH. Pac1 was determined to be a functional homolog of PacC by complementation of an *A. nidulans* PacC null strain with *Pac1* (Rollins and Dickman, 2001). Importantly, gene replacement of Pac1 resulted in the inability of *S. sclerotiorum* to cause appreciable disease symptoms on *Arabidopsis* and tomato (Rollins, 2003). These results suggest that ambient pH is a regulatory cue for processes linked to development and virulence.

How might pH modulation specifically affect these phenotypes? Inhibitor studies suggested involvement of

mitogen-activated protein kinases (MAPKs) in sclerotial development. A highly conserved homolog of extracellular signal-regulated protein kinase-type MAPKs from *S. sclerotiorum* (Smk1) was identified and shown to be required for sclerotial development (Chen et al., 2004). The transcription of *smk1* and MAPK enzyme activity were induced dramatically during the production of sclerotial initials. Notably, *smk1* transcript levels were highest under acidic pH conditions. Treatment with hydrochloric acid induced *smk1* transcription in the same way as treatment with OA did; however, this did not occur with potassium oxalate, suggesting that Smk1 regulates sclerotial development via a pH-dependent signaling pathway. Thus, proper regulation of OA is required for sclerotial development.

In a separate study, addition of cyclic AMP (cAMP) inhibited *Smk1* transcription, MAPK activation, and sclerotial development. These effects were hypothesized to be mediated by cAMP-dependent protein kinase (PKA), a major intracellular receptor for cAMP. To test this, a *pka* catalytic subunit gene was cloned and disrupted. Mutant strains were cAMP responsive and pathogenic and had wild-type levels of PKA activity, thus questioning the importance of PKA dependency. Phylogenetic analyses of *Sclerotinia* PKA with other fungal *pka* genes and encoded peptides suggest that filamentous fungi possess two *pka* paralogs (Jurick et al., 2004). Thus, the roles of PKAs in these processes still await confirmation and the mechanism(s) by which cAMP inhibits MAPK remains unclear. Expression of a dominant negative form of *ras*, an upstream activator of the MAPK pathway, also inhibited sclerotial development and MAPK activation, suggesting that a conserved Ras and MAPK pathway is required for sclerotial development. Evidence from bacterial toxins that specifically inhibit the activity of small GTPases suggested that repressor activator protein (Rap-1) or Ras is involved in the negative effect of cAMP. The Rap-1 inhibitor, GGTI-298, restored MAPK activation in the presence of cAMP, suggesting that Rap-1 is responsible for cAMP-dependent MAPK inhibition. Importantly, inhibition of Rap-1 is able to restore sclerotial development that is blocked by cAMP. These results suggested a novel mechanism involving the requirement of the Ras and MAPK pathway for sclerotial development that is negatively regulated by the PKA-independent cAMP signaling pathway. Cross talk between these two pathways is mediated by Rap-1 (Chen and Dickman, 2005). Thus, *S. sclerotiorum* can coordinate environmental signals (such as pH) to trigger a signaling pathway mediated by Smk1 to induce sclerotium formation and this pathway is negatively regulated by cAMP.

ROS-Fungal Development

During the course of these studies, intriguing and distinct links between potential signaling properties of reactive oxygen species (ROS), pathogenesis, and sclerotial development were noted. In all eukaryotes examined, ROS are produced during normal cellular metabolism. It is now evident that low, nonlethal concentrations of ROS can function beneficially as regulatory molecules in cell-signaling pathways. The importance of the redox "climate" in fungal growth and development has been suggested by Hansberg and Aguirre (1990), who proposed that hyperoxidant states are a primary driving force for the differentiation states in microorganisms, including fungi. Evidence implying that sclerotial differentiation is affected by oxidative stress includes the observation that hydroxyl radical scavengers inhibit sclerotial differentiation and growth of *S. sclerotiorum* and *Rhizoctonia solani* (Georgiou et al., 2000). Additional evidence for the involvement of ROS in sclerotial metamorphosis (in *Sclerotium rolfsii*, *R. solani*, *S. sclerotiorum*, and *Sclerotium minor*) is provided by the decrease in sclerotial numbers in culture exposed to the O_2^- scavenger Tempol, a mimetic of the antioxidant enzyme SOD (Georgiou et al., 2000). Consistent with these observations, an *S. sclerotiorum* SOD gene was knocked out; sclerotium development was blocked and virulence was reduced (S. Veluchamy and M. Dickman, unpublished). DCF (2′,7′-dichlorofluorescein) fluorescence showed a dramatic increase of ROS as sclerotial initials form. Chemical inhibition of ROS by diphenyliodonium (DPI), an NADPH oxidase inhibitor, or *N*-acetyl cysteine, an ROS scavenger, suppressed sclerotial development while having no overt effects on fungal growth. Thus, while the role of ROS as signal molecules during sclerotial development has yet to be completely established, the involvement of ROS signaling during this process is emerging.

Regulation by ROS coupled with OA and pH signaling may prove to be an important strategy by which *S. sclerotiorum* senses its environment and achieves pathogenic success (Dickman, 2007a, 2007b). One of the earliest resistance responses mounted by infected plant tissues against an invading microbe is the oxidative burst, that is, the controlled release of O_2^- and H_2O_2 at the site of pathogen ingress (e.g., Bolwell, 1999; Torres and Dangl, 2005). The oxidative burst is thought to be required for several subsequent defense responses and is expressed in most if not all plant species (Wojtaszek, 1997). We hypothesized that OA might impact this primary response and early plant defense responses. Of relevance is the observation that the oxidative burst is suppressed at low pH (Legendre et al., 1993). Because the release of OA lowers the pH, OA could enhance fungal pathogenicity by inhibiting the oxidative burst of the host plant. Biochemical studies confirmed this to be the case (Cessna et al., 2000). Moreover, OA^- mutants of *S. sclerotiorum*, which are nonpathogenic, were unable to inhibit plant ROS (defense) induction. Importantly, OA blocked the oxidative burst pathway even at the optimal pH of the pathway. Taken together, these data revealed a previously unrecognized function of OA synthesis and secretion by *S. sclerotiorum*, namely, the ability to suppress ROS generation and thereby compromise plant defense responses. Perhaps paradoxically, OA can also increase ROS accumulation in plants via the enzyme oxalate oxidase, which is found in all monocot plants (know as germin) and several dicots. Oxalate oxidase converts OA to hydrogen peroxide (H_2O_2), and various forms of this enzyme have been used in transgenic strategies for plant protection from *S. sclerotiorum*-induced disease (e.g., Donaldson et al., 2001; Hu et al., 2003). Examination of the genome sequence of *S. sclerotiorum* reveals an open reading frame with potential oxalate oxidase activity. It is tempting to speculate that this observation may have biological relevance. As mentioned, when sclerotial initials develop, elevated levels of ROS (generally H_2O_2) are clearly present. Moreover, the *smk1* gene encoding a *Sclerotinia* MAPK required for sclerotial formation is transcriptionally activated by H_2O_2. Taken together, these data suggest that ROS are spatially and temporally regulated and are important for proper sclerotial development and disease. The endogenous source of ROS is currently a key question; they may originate from a fungal oxalate oxidase or from alternative sources such as peroxidases or NADPH oxidases.

ROS-Programmed Cell Death and Disease

By definition, necrotrophic fungi require dead tissue for growth. The manner by which these fungi achieve host cell

death has recently been examined in detail. An indication that the process of host cell death may be more than just direct fungal killing is illustrated by the fact that several cytoprotective, antiapoptotic genes, when expressed in transgenic tobacco, conferred resistance to *S. sclerotiorum* but were not fungitoxic; in other words the fungus grew on the plant surface but was unable to cause disease (and unable to induce cell death; see below).

Programmed cell death (PCD) is an intentional cellular suicide that is genetically based. The result of PCD is the orderly removal of unwanted, unneeded, used, or pathological cells and under normal homeostatic conditions, is of benefit to the organism. In contrast, necrosis is an accidental cell death that is independent of genetic background. PCD is essential for proper growth and development of all eukaryotes. PCD may be viewed as a continuum ranging from apoptosis to autophagy, and this topic and the distinction between these processes have been extensively discussed (Williams and Dickman, 2008, and references therein), although there are many gray areas and much overlap between these death regimes.

Since cell death of host tissue is requisite for disease and successful colonization, interference with the cell death process could presumably block the success of this type of pathogen. By use of a transkingdom approach, several animal cell death regulatory genes were transferred into tobacco. These genes have a common antiapoptotic/cytoprotective function. Transgenic tobacco plants expressing Bcl-2, Bcl-xl, CED-9, and Op-IAP were resistant to cell death and disease caused by *S. sclerotiorum*, *B. cinerea*, and other necrotrophic fungi (Dickman et al., 2001; M. Dickman, unpublished data). DNA laddering and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) reacting plant cells, markers indicative of a programmed cell death, were observed in wild-type inoculated plants during fungal pathogenesis. These features were not observed in resistant, transgenic plants. It should be noted that these results were obtained in experiments using wild-type *S. sclerotiorum* having its full arsenal of weapons, including OA and hydrolytic enzymes. Based on these observations, it was concluded that if cell death is impeded, disease is prevented. By contrast, the hypersensitive response of plants to certain biotrophic pathogens is a programmed cell death resulting in a resistant phenotype (Mur et al., 2008). These data suggest that cell death control may be key in the outcome of a given plant-microbe interaction and is context dependent.

The question remains however, as to how *S. sclerotiorum* achieves its broad-based pathogenic success by cell death regulation. When administered to plant tissue, fungal extracts mimicked disease symptoms, i.e., cell death. These extracts also induced apoptosis-like features in plant cells. There were no differences in cell death response when boiled extracts were used, strongly suggesting that the elicitor for cell death is not proteinaceous. Oxalate is an obvious possibility, and indeed oxalate (both as an acid or in salt formulation) induced a PCD response in plant tissue. This response exhibited features associated with mammalian apoptosis, including DNA laddering and TUNEL reactive cells. The induction of PCD by OA is independent of the pH-reducing abilities of this organic acid, thus differing from its role in sclerotial development, where pH decrease was necessary for development. Further support is shown by the facts that fungal mutants deficient in OA production are nonpathogenic and apoptosis-like characteristics are not observed following plant inoculation. Thus, OA induces a PCD pathway in plant tissue that is required for disease development (Kim et al., 2008).

What is the mechanism of action for OA-directed PCD? One possibility is the generation of ROS. DAB (3,3′-diaminobenzidine) staining (indicative of H_2O_2 production) showed that increased ROS levels were present in plant tissue following treatment with oxalate. When this ROS induction is inhibited (e.g., by DPI), plant apoptosis-like cell death induced by OA does not occur. Oxalate-induced ROS in the plant correlates with PCD. Thus, oxalate can function as an elicitor of PCD. We conclude that OA secreted by *S. sclerotiorum* is an elicitor of PCD in plants and is responsible for induction of apoptosis-like features in the plant during disease development. This PCD is essential for fungal pathogenicity and involves ROS. Thus, OA appears to be central to ROS signaling, causing PCD-like cell death of the host, which is consistent with its importance in establishing a necrotrophic relationship. OA thus appears to subvert the plant host by inappropriately triggering pathway(s) responsible for PCD.

B. CINEREA

B. cinerea (Fig. 2a) is closely related to *S. sclerotiorum*. It also has a broad host range, of over 200 plant species, comprising mainly dicots. Diseases caused by *B. cinerea* occur in important crop plants in all temperate climate zones, both during plant cultivation and on harvested co7mmodities, often during storage. Annual losses of crops from diseases caused by *B. cinerea* are of the same order of magnitude as those caused by *S. sclerotiorum*, if not greater. *B. cinerea* infection is difficult to control with chemicals, because of the emergence of fungicide-resistant genotypes, some of which display multidrug resistance (Leroux et al., 2002). Only recently have examples of genetically determined (partial) resistance to *B. cinerea* in tomato (Finkers et al., 2007a, 2007b) and *Arabidopsis thaliana* (Denby et al., 2004; Rowe and Kliebenstein, 2008) been reported.

Although molecular tools became available for many filamentous fungi from the late 1970s onwards, it was not until the 1990s that efforts were undertaken to study *B. cinerea* at the molecular genetic level. The quantitative nature of pathogenicity and host susceptibility as well as the great genetic diversity and reported genetic instability of *B. cinerea* isolates has long inhibited molecular geneticists from initiating studies of this pathogen. Molecular tools have now been developed for stable transformation of protoplasts (Hamada et al., 1994), *Agrobacterium*-mediated transformation of germlings (Rolland et al., 2003), gene replacement (van Kan et al., 1997), and RNA interference-based knockdown of gene expression (Patel et al., 2008).

Sclerotia and Apothecia

Like *S. sclerotiorum*, *B. cinerea* produces sclerotia that may persist in soil in a dormant state, but their survival has not been extensively studied in the field. The environmental signals that affect sclerotium formation in *B. cinerea* include low light, nutrient limitation, temperature, and pH. Sclerotial development in *B. cinerea* has not been studied as extensively as in *S. sclerotiorum*, but the morphology and development of sclerotia of the two fungi are very similar. Gene disruption of the MAPK gene *Bcsak1* resulted in enhanced formation of sclerotia (Segmüller et al., 2007), whereas replacement of the NADPH oxidase genes *bcnoxA* and *bcnoxB* resulted in loss of sclerotium development (Segmüller et al., 2008).

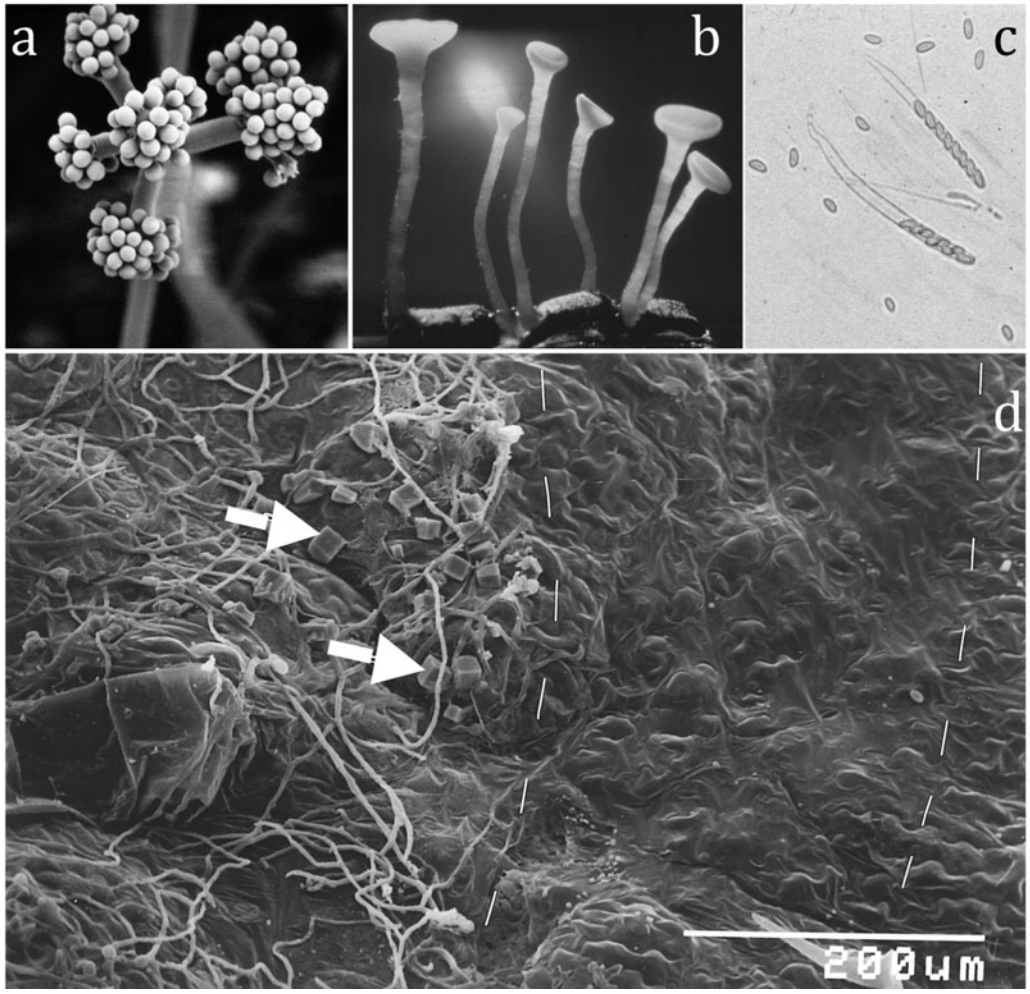

FIGURE 2 Life stages of *B. cinerea* (*Botryotinia fuckeliana*). (a) *B. cinerea* conidiophore with mature conidia in situ (low-temperature scanning electron microscopy). (b) Apothecia of *B. fuckeliana*, ~10 weeks after spermatization. (c) Two *B. fuckeliana* asci each containing eight ascospores, surrounded by ascospores released from damaged asci. (d) Scanning electron micrograph of calcium oxalate crystals (indicated by arrows) in tomato leaves infected by *B. cinerea*. The white dashed line in the center of the picture indicates the border of the lesion between the external mycelium surrounded by crystals (on the left-hand side) and a concentric zone of collapsed epidermal cells (on the right-hand side). Both zones are colonized by mycelium, growing below the epidermis. The white dashed line at the right-hand side of the picture represents the border between the colonized area and noninvaded leaf tissue. Panels a through c reproduced from Williamson et al., 2007, with permission from Blackwell Publishers. Panel d reproduced from Prins et al., 2000b, with permission.

B. cinerea sclerotia can either germinate asexually to form hyphae and conidia or germinate carpogenically to start sexual development (Faretra et al., 1988), leading to the production of apothecia (Fig. 2b) from which ascospores (Fig. 2c) are liberated. Ascospores may serve as inoculum, especially in early spring, when apothecia are thought to develop on sclerotia embedded in crop debris in the soil. Population studies provided evidence that *B. cinerea* frequently reproduces sexually (Giraud et al., 1997, 1999; Fournier et al., 2005), even though observations of apothecia in the field have been published only five times since 1865. *B. cinerea* is generally heterothallic, but there are numerous examples of strains that are able to mate with both mating-type reference strains (van der Vlugt-Bergmans et al., 1993; Faretra and Pollastro, 1996), as well as able to self-fertilize, defining such strains as homothallic. Remarkably, several homothallic strains possess a single mating-type gene (most often the high-mobility-group domain gene) and there is no evidence for presence of the complementary MAT gene (J. van Kan, unpublished data). This phenomenon is yet poorly understood.

Oxalate

Like *S. sclerotiorum*, *B. cinerea* secretes significant amounts of OA (Fig. 2d), and it has long been considered that OA would be as important for virulence of *B. cinerea* as it is for *S. sclerotiorum* (Godoy et al., 1990). Especially, OA secretion might stimulate enzymes secreted by *B. cinerea* during invasion of plant tissues (e.g., polygalacturonases, proteases, laccases, and peroxidases), all of which have maximal

activities at low pH (Manteau et al., 2003) and several of which are important for virulence (see below). Yet, unlike *S. sclerotiorum*, OA appears not to be essential for virulence of *B. cinerea*. Gene replacement of the oxaloacetate hydrolase gene *Bcoah1* yielded mutants that do not produce detectable levels of OA in vitro (Han et al., 2007) or in planta (F. Stefanato and H. Schoonbeek, unpublished data). Virulence of the *Bcoah1*-deficient mutant was unaffected on tomato leaves (Han et al., 2007) but reduced to different degrees on other host species or tissue types (Stefanato and Schoonbeek, unpublished).

Secreted Enzymes Involved in Tissue Penetration and Decomposition

B. cinerea differentiates appressoria that breach the cuticle by means of a penetration peg (Tenberge, 2004) in a process that requires the membrane-associated protein BcPLS1 (Gourgues et al., 2004), whose function remains to be resolved. *B. cinerea Bcpls1*-deficient mutants form appressoria of normal structural appearance, but they cannot penetrate an intact plant surface (Gourgues et al., 2004). Penetration of the plant surface does not involve the generation of high turgor and is presumably mediated by secreted fungal enzymes. One enzyme that plays an early role in host surface penetration by appressoria is a secreted superoxide dismutase (BcSOD1) (Rolke et al., 2004). An oxidative burst occurs during cuticle penetration (Schouten et al., 2002), and BcSOD1 may contribute to this process. Deletion of the *Bcsod1* gene led to reduced virulence on multiple hosts (Rolke et al., 2004). The source of superoxide acting as a substrate for BcSOD1 remains to be identified. NADPH oxidases (Nox) were considered to be candidates for superoxide production. Functional analysis of the two *nox* genes present in the *B. cinerea* genome (*bcnoxA* and *bcnoxB*) showed that they both have significant impact on virulence (Segmüller et al., 2008). While *bcnoxB* mutants showed a delay in primary lesion formation, due to an impaired formation of penetration structures, *bcnox*A mutants penetrated host tissue in the same way as the wild type but colonized the host tissue much slower (Segmüller et al., 2008). Double mutants were aberrant both in penetration and in colonization of plant tissue and hence were almost nonpathogenic. The *bcnoxR* gene (encoding a regulatory subunit of the Nox complex) was also functionally characterized. The phenotype of *bcnoxR* mutants was identical to that of *bcnoxA bcnoxB* double mutants, providing evidence that BcnoxR is involved in activation of both Bcnox enzymes. However, the impact of BcnoxR enzymes on the ROS status in planta has yet to be determined. Similar to what was observed in *S. sclerotiorum*, *bcnoxA* and *bcnoxB* mutants were deficient in formation of sclerotia (Segmüller et al., 2008), emphasizing the role of ROS in development.

Deletion of a cutinase gene and a lipase gene, either separately or together, did not detectably reduce virulence (van Kan et al., 1997; Reis et al., 2005). The genome of *B. cinerea*, however, contains multiple additional cutinase and lipase genes, and unraveling the role of these enzyme families in pathogenesis requires further study. Upon breaching the cuticle, the penetration peg often grows into the anticlinal wall of an epidermal cell, which is rich in pectin. Invasion of this layer involves the action of pectinases, especially the endopolygalacturonase BcPG2. Mutants in which the *Bcpg2* gene was deleted showed a delay in primary lesion formation on bean and tomato leaves (Kars et al., 2005a). *B. cinerea* contains six endopolygalacturonase genes (Wubben et al., 1999), and their expression during infection varies depending on the host, tissue type, and incubation conditions (ten Have et al., 2001), suggesting functional versatility. Deletion of two endopolygalacturonase genes, separately, resulted in a pronounced reduction of lesion growth rates on several host plants (ten Have et al., 1998; Kars et al., 2005a), whereas single-deletion mutants in the other four endopolygalacturonase genes were not affected in virulence (Kars, 2007).

It is generally considered that endopolygalacturonases cannot efficiently hydrolyze methylated pectin; hence, demethylation by pectin methylesterases (PMEs) presumably precedes and facilitates pectin hydrolysis by endopolygalacturonases. This assumption implies that PMEs could be important for fungal growth on highly methylated pectin and for virulence on plant tissues with highly methylated pectin (such as leaves), but not on tissues with low pectin methylation (such as fruit). The virulence of single and double mutants in two *Bcpme* genes in strain B05.10, however, was identical to that of the wild type (Kars et al., 2005b). Surprisingly, the wild-type strain and the *Bcpme*-deficient mutants grew better on 75% methylated pectin than on nonmethylated polygalacturonic acid, suggesting that pectin demethylation by PMEs is not important for its depolymerization in vivo by endopolygalacturonases (Kars et al., 2005b).

Besides pectinases, other types of cell wall-degrading enzymes produced by *B. cinerea* have been studied. Deletion of a β-1,4-xylanase gene delayed lesion formation and reduced lesion outgrowth by more than 70% (Brito et al., 2006), whereas the deletion of a cellulase gene did not affect virulence (Espino et al., 2005).

Phytotoxic Compounds

B. cinerea can produce a spectrum of phytotoxic metabolites of low molecular weight, as well as phytotoxic proteins. The best-studied phytotoxic metabolite is botrydial, which accumulates in infected plant tissue at considerable levels (Deighton et al., 2001). The biosynthetic pathway for botrydial has been resolved (Colmenares et al., 2002), and genes involved in its biosynthesis appear to be organized in a cluster containing at least two cytochrome P450 monooxygenase genes, as well as a terpene cyclase gene (Siewers et al., 2005). Deletion of one of the cytochrome P450 monooxygenase genes, named *Bcbot1*, in three different strains resulted in severe reduction of virulence in one strain but not in the others (Siewers et al., 2005). Deletion of the *Bcbot2* gene, encoding a sesquiterpene cyclase, caused the same phenotype as the *Bcbot1* mutant (Pinedo et al., 2008). These observations suggest that certain strains (e.g., T4) strictly require botrydial to kill host cells, while others (e.g., B05.10) can produce additional toxins, such as botcinolide (Reino et al., 2004), of which the chemical structure was later revised and which was renamed botcinic acid (Tani et al., 2006). The biosynthetic pathway of botcinic acid remains to be resolved but may involve a polyketide synthase (I. Collado and B. Tudzynski, personal communication).

Besides phytotoxic metabolites, *B. cinerea* can produce at least three distinct phytotoxic proteins, i.e., two NEP1-like proteins (Staats et al., 2007) and a Snodprot homolog named *Bcspl1* (Chagué et al., 2006; Kunz et al., 2006). *B. cinerea* NEP1-like proteins were found to associate with plant plasma membranes as well as the nuclear envelope, and the proteins can cause host cell death by a combination of apoptotic and

necrotic mechanisms (Schouten et al., 2008). Neither of the NEP1-like proteins is important for virulence (Y. Cuesta Arenas and J. van Kan, unpublished data).

ROS-Programmed Cell Death and Disease

Until a few years ago, it was considered that host plants play a rather passive role in the interaction with necrotrophic pathogens. It was assumed to be sufficient for the pathogen to expose the host to phytotoxic compounds that trigger the generation of an oxidative burst that would induce plant cell death and thereby promote pathogen invasion. *B. cinerea* is able to secrete phytotoxic compounds, as well as enzymes that can directly generate ROS at the plant-fungus interface, such as glucose oxidase and superoxide dismutase (Rolke et al., 2004) or glyoxal oxidase (van Kan, unpublished). Collectively, the oxidative stress imposed by these compounds leads to the accumulation of free radicals, both at the host-pathogen interface and at some distance from the invading *B. cinerea* hyphae, culminating in plant lipid peroxidation and the depletion of antioxidants, causing massive perturbation of the redox status in and around the infected tissue, thereby promoting disease progress (reviewed by Lyon et al., 2004).

Recent research has revealed that the host plays a more active role and the interactions between plants and necrotrophic fungi are more subtle in their attack than previously appreciated. The ability to induce PCD appears to play a pivotal role in the success of *B. cinerea*. Infection of *Arabidopsis* by *B. cinerea* induces cell death concomitant with nuclear condensation and expression of the HR-specific gene *Hsr203* (Govrin and Levine, 2000). Expression of *Hsr203* and activation of metacaspase activity, both suggestive of the occurrence of programmed cell death, were observed in *B. cinerea*-infected tomato (Hoeberichts et al., 2003). In *Botrytis*-infected *Arabidopsis* leaves, the oxidative burst comprises the simultaneous production of H_2O_2 and nitric oxide as well as the formation of proteolytic, autophagosome-like vesicles at the host-pathogen interface (van Baarlen et al., 2007). Mutations in *Arabidopsis* that promote cell death increase susceptibility, while mutations that delay cell death increase resistance to *B. cinerea* (Govrin and Levine, 2000; van Baarlen et al., 2007). The observation that PCD is an important determinant in the interaction of *B. cinerea* with host plants was corroborated by the fact that transgenic plants expressing heterologous antiapoptotic genes display an increased resistance to *S. sclerotiorum* and *B. cinerea* (Dickman et al., 2001) as discussed above. It remains to be established whether the phytotoxic metabolites and proteins secreted by *B. cinerea* are inducers of PCD (apoptosis) rather than direct-acting toxins causing disorganized death (necrosis).

ROS Metabolism by *B. cinerea*

While *B. cinerea* continuously generates oxidative stress in and around the plant tissues that it invades, the fungus obviously must be able to cope with a hostile, oxidative environment. *B. cinerea* may convert superoxide radicals into H_2O_2 by means of a secreted superoxide dismutase, BcSOD1 (Rolke et al., 2004), whereas the resulting H_2O_2 can be neutralized by an extracellular catalase (Schouten et al., 2002). When H_2O_2 nevertheless enters the fungal hyphae, it might be counteracted by an intracellular catalase (van der Vlugt-Bergmans et al., 1997) or by a combination of a glutathione *S*-transferase (Prins et al., 2000a) that conjugates H_2O_2 to glutathione and an ABC transporter (possibly BcatrO [Pane et al., 2008]) that expels the oxidized glutathione conjugate from the cytoplasm.

Signaling

Signaling processes play an important role in virulence of *B. cinerea*. Successful infection requires germination of conidia on the plant tissue, differentiation of infection structures, and invasion of plant tissue that contains a spectrum of distinct antimicrobial defense molecules. These processes require an adequate perception of the physical and chemical environment and strategic decisions on the expression of genes at an appropriate time and place. *B. cinerea* contains all the signaling pathway components typical for saprophytic and pathogenic ascomycetes, including receptors similar to G-protein-coupled receptors (Schulze Gronover et al., 2005), a heterotrimeric G protein complex (Schulze Gronover et al., 2001), histidine kinases (Cui et al., 2002; Catlett et al., 2003; Viaud et al., 2006), three MAPK cascades (Zheng et al., 2000; Döhlemann et al., 2006; Segmüller et al., 2007; Rui and Hahn, 2007), RAS- and RAC-type GTPases (Schumacher et al., 2008a), the cAMP signaling cascade (Klimpel et al., 2002; Schumacher et al., 2008a), calcineurin (Viaud et al., 2003; Schumacher et al., 2008b), and phospholipase C (Schumacher et al., 2008b). Functional analysis has been performed for at least 25 of these genes. Mutants in signaling pathways can be affected in particular stages of pathogenesis, either in germination on the plant, in surface penetration, in primary lesion formation, or in lesion expansion. Some of the reduced virulence phenotypes are associated with defects in germination, hyphal morphology, or growth rate. Extensive discussion of the complex roles of the various signaling pathways mentioned above in virulence of *B. cinerea* is beyond the scope of this chapter. Readers are referred to a review by Williamson et al. (2007) and more recent original publications for a detailed description and discussion of this subject.

A. BRASSICICOLA

A. brassicicola infects a broad range of species within the Brassicaceae plant family and is taxonomically distinct from both *S. sclerotiorum* and *B. cinerea*. *A. brassicicola* is a member of the Dothideomycetes, and this group contains many other important plant pathogens found in the genera *Cochliobolus*, *Pyrenophora*, *Leptosphaeria*, *Cladosporium*, *Stagonospora*, and *Mycosphaerella*. Importantly, as of January 2009, genome sequencing projects have been completed for at least one if not several plant pathogenic species in these genera, including *A. brassicicola*. Whole-genome comparisons between these fungi are currently under way and should shed light on conserved and unique pathogenicity mechanisms employed by the Dothideomycetes. Since efficient gene knockout methods were not optimized for *A. brassicicola* until approximately 2005 (Cho et al., 2006), the analysis of pathogenicity mechanisms has not been as detailed as analyses for *S. sclerotiorum* and *B. cinerea*. Nevertheless, considerable progress has been made over the last several years, with over 100 genes functionally evaluated with respect to pathogenicity and development in certain cases. Moreover, *A. brassicicola* is very often used in studies with the model host plant *Arabidopsis*, which is a member of the Brassicaceae plant family, thus making this fungus an excellent choice for studying coevolutionary aspects of host-microbe interactions, since genome sequencing of both host and pathogen has now been completed.

A. brassicicola-Brassicaceae Pathosystem

The crucifer plant family Brassicaceae comprises approximately 3,500 species in 350 distinct genera. The most

important crop species from an economic perspective are found within a single genus, *Brassica*. The crop species include *B. oleracea* (vegetables), *B. rapa* (vegetables, oilseeds, and forages), *B. juncea* (vegetables and seed mustard), and *B. napus* (oilseeds) (Westman et al., 1999). *A. brassicicola* causes black spot disease on virtually every important *Brassica* species and is of worldwide economic importance (Sigareva and Earle, 1999a; Westman et al., 1999; Rotem, 1994). For example, black spot has been reported to cause 20 to 50% yield reductions in crops such as canola or rape (Rotem, 1994). Similar to other *Alternaria*-associated diseases, black spot appears on the leaves as necrotic lesions, which are often described as black and sooty with chlorotic yellow halos surrounding the lesion sites (Rotem, 1994). *A. brassicicola* can infect all parts of the plant, including pods, seeds, and stems, and is of particular importance as a postharvest disease (Rimmer, 1995). The necrotrophic nature of *A. brassicicola* typically leads to extensive damage of the plant with seedlings seldom surviving an attack (Rimmer, 1995; Humpherson-Jones, 1985) (Fig. 3). *A. brassicicola* is often seed transmitted, although transmission by insects has also been reported (Dillard et al., 1998). Between 1976 and 1978, 86% of the commercial *Brassica* seed produced in the United Kingdom was contaminated with *A. brassicicola* (Maude and Humpherson-Jones, 1980). The primary disease consistently affecting the cabbage seed industry in the United States is also caused by *A. brassicicola* and the related species *A. brassicae*. Infection decreases seed yield, quality, and germination. Spread of the disease during the growing season is typically by rain- and wind-dislodged spores. Optimal conditions for sporulation and infection include a minimum wet period of 13 h and ambient temperatures of 20 to 30°C (Rotem, 1994; Humpherson-Jones and Phelps, 1989). Consequently, black spot disease has been of particular importance in regions of the world with cool, wet weather during the growing season, such as in the United Kingdom, Thailand, and the northeastern United States (Pattanamahakul and Strange, 1999).

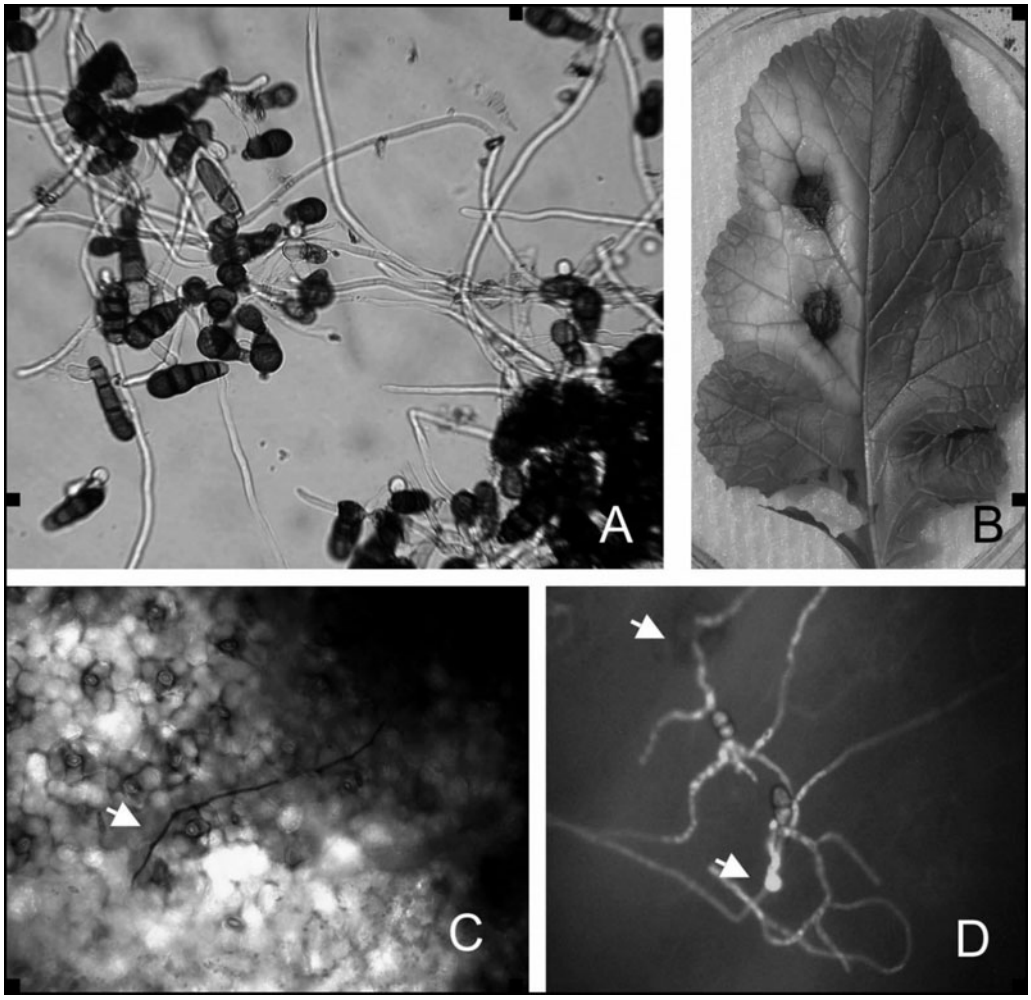

FIGURE 3 *A. brassicicola*. (A) Germinating spores of *A. brassicicola* in vitro. (B) Black spot symptoms on cultivated mustard, *B. juncea*. (C) Trypan blue staining of infected cabbage tissue. The arrow points to an invasive hypha. Note dark blue staining of tissue surrounding hypha, suggesting damaged walls and membranes of host cells due to the action of toxic secreted proteins and metabolites. (D) GFP-tagged *A. brassicicola* germinating on a cabbage leaf. Arrows depict entry through stomata and appressorium-like structures. Reprinted from Lawrence et al. (2008).

High levels of resistance to *A. brassicicola* have been reported in weedy members of the Brassicaceae, such as *A. thaliana*, *Camelina sativa*, and *Capsella bursa-pastoris*, but no satisfactory source of resistance has been identified among cultivated *Brassica* species (Conn et al., 1988; Sigareva and Earle, 1999a, 1999b; Westman et al., 1999). Of the very few *Brassica* species or breeding lines that have been reported to possess a minor-to-moderate level of resistance, the genetic basis appears to involve additive and dominant gene action (King, 1994). Several *Arabidopsis* mutants have been reported to have enhanced susceptibility to *A. brassicicola* (see reviews by Thomma, 2003; and Glazebrook, 2005). Very recently, individual ecotypes, such as Dijon G, have been shown to exhibit a compatible interaction to *A. brassicicola* (Mukherjee et al., 2009). Thus, opportunities now exist to unravel the mechanisms of both resistance and susceptibility, by employing select *Arabidopsis* mutants, ecotypes, and recombinant inbred-line populations. In addition to *S. sclerotiorum* and *B. cinerea*, *A. brassicicola* represents one of the few bona fide necrotrophic pathogens of *Arabidopsis* and thus can be used in conjunction with the considerable experimental power that *Arabidopsis* offers (for a review, see Glazebrook, 2005). In addition, crop improvement of economically important plants, such as canola, will likely benefit from studies of interactions between *A. brassicicola* and *Arabidopsis*.

A. brassicicola Functional Genomics

A transformation method was established for *A. brassicicola* in order to generate targeted gene disruption mutants at 80 to 100% efficiency for diverse genes using "linear minimal element" constructs (Cho et al., 2006). This was the first study in which polyethylene glycol-mediated protoplast transformation of this fungus was reported to be successful. Targeted gene disruption was accomplished using a single homologous recombination event. The targeting efficiency was consistently high for small constructs harboring 250-bp homologous sequences corresponding to target genes. Shorter sequences have not been evaluated. In a recent regulatory gene study described in more detail below, the transformation method was used for replacement of target genes by the hygromycin B phosphotransferase cassette, using linear replacement constructs containing ~1-kb genomic sequences that flank the target gene. The efficiency of targeted gene disruption or gene replacement in *A. brassicicola* is high when compared to that observed in other fungi with sequenced genomes. This high efficiency makes targeted functional genomics in *A. brassicicola* feasible and amenable to high-throughput experiments. The majority of the >100 targeted gene mutants produced thus far have shown little or no reduction of virulence (C. B. Lawrence, Y. Cho, K. Kim, H. X. Dang, C. M. Larota, D. C. Scott, G. Santopietro, and S. W. Park, unpublished data). The genes studied were selected based on in planta expression levels or machine-annotated features of interest (e.g., histidine kinases, secondary metabolite biosynthesis, transcription factors, cell wall-degrading enzymes, etc.). Several genes identified in these studies are intriguing, and exciting exceptions as described below.

Cell Wall-Degrading Enzymes

An examination of the role of cutinase genes in *A. brassicicola* pathogenesis was conducted previously (Yao and Köller, 1994, 1995). In these studies, biolistic transformation (gene gun) was used to disrupt the CUTAB1 gene. Disruption of CUTAB1 affected saprophytic growth on cutin as a sole carbon source but had no significant effect on pathogenicity. An extracellular lipase was produced by *A. brassicicola* in vitro, and supplementation of the inoculum with antilipase antibodies significantly reduced disease on cauliflower leaves (Berto et al., 1999). However, disruption of four individual, predicted *A. brassicicola* lipase genes expressed during plant infection did not result in reduced virulence on cabbage (Cho et al., 2006; Lawrence et al., unpublished). These results suggest functional redundancy of lipases in pathogenicity, especially given the 12 to 18 predicted lipases in the *A. brassicicola* genome (Lawrence et al., unpublished). It is possible that the polyclonal antilipase antibodies used by Berto et al. (1999) blocked the action of multiple lipases simultaneously or blocked the action of an as-yet-unidentified gene product. Besides lipases, several other genes have been studied, including an endoxylanase, cellulase, and pectate lyase, but no defects in virulence have been observed (C. Lawrence, unpublished data).

Secondary Metabolites

The genus *Alternaria* has traditionally been reported to be a prolific producer of secondary metabolites and, in particular, phytotoxins (Rotem, 1994). All of the plant pathogenic *Alternaria* species to date have been reported to produce host-specific toxins and/or non-host-specific toxic substances, having very diverse biochemical structures (Rotem, 1994; Thomma, 2003). For many of the plant pathogenic *Alternaria* species, toxin production has been clearly demonstrated to be essential in enabling disease development on a particular host(s). Several toxins have been reported to be produced by *A. brassicicola*. AB-toxin is proteinaceous and, interestingly, produced only when on host plants (Otani et al., 1998). Moreover, *A. brassicicola* produces other toxic substances including diterpenoid, fusicoccin-like compounds, some of which have been termed brassicicenes (McKenzie et al., 1988; Cooke et al., 1997; MacKinnon et al., 1999). Recently the brassicicene biosynthetic gene cluster has been identified, but its role in pathogenicity has yet to be elucidated (Minami et al., 2009). Other secondary metabolites, nonspecific but possibly host-selective toxins, have recently been described (Pedras et al., 2009). Lastly, *A. brassicicola* has been shown to produce depudecin, an 11-carbon linear polyketide that acts as an inhibitor of histone deacetylase. A chemically unrelated histone deacetylase inhibitor, HC-toxin, was earlier shown to be a major virulence factor in the interaction between *Cochliobolus carbonum* and its host, maize (Walton, 1996). Recently, the polyketide synthase-based biosynthetic gene cluster responsible for depudecin production was identified and functionally characterized but was found to be only a minor virulence factor (Wight et al., 2009).

One major area of interest lies in the area of secondary metabolite biosynthesis, stress tolerance, and development. A nonribosomal peptide synthase gene (*NPS6*) in *Cochliobolus heterostrophus* and *A. brassicicola* was found to direct the biosynthesis of a siderophore metabolite important for oxidative stress tolerance and pathogenicity (Oide et al., 2006). Another nonribosomal peptide synthase gene (*AbNPS2*) was found to be important for cell wall integrity, conidial viability, and virulence of aged spores of *A. brassicicola* (Kim et al., 2007). The secondary metabolite synthesized via *AbNPS2* has yet to be characterized. Clearly more research is needed to further characterize secondary metabolite biosynthetic genes and their role in pathogenicity and fungal development.

Signaling and ROS

Another area ripe for exploration in the *A. brassicicola*-Brassicaceae pathosystem is fungal signal transduction mechanisms and the role of ROS. Disruption of the Fus3/Kss1 MAPK homolog (*Amk1*) in *A. brassicicola* resulted in a complete loss of pathogenicity, as observed in other fungi (Cho et al., 2006, 2007). Interestingly, in the latter study it was shown that addition of long polypeptides such as tryptone partially restored pathogenicity to the mutants. In contrast to the MAPK mutants in other phytopathogenic fungi, *A. brassicicola amk1* disruption mutants were capable of partially infecting wounded tissues. In another recent study, targeted gene deletion mutants in 21 putative regulatory genes were produced in *A. brassicicola* (Cho et al., 2009). In this study, the SNF1 kinase, several histidine kinases, and transcription factor homologs such as Ste12, CreA, PacC, and Pro1 were subjectively selected from the annotated *A. brassicicola* genome. The SNF1 kinase plays a central role in carbon catabolite repression in *Saccharomyces cerevisiae* (Palecek et al., 2002). Deletion of homologous genes in *C. carbonum* and *Fusarium oxysporum* resulted in reduced virulence due to decreased expression of cell wall-degrading enzyme genes under derepressive conditions, including growth in media utilizing complex carbohydrates as a carbon source (Ospina-Giraldo et al., 2003; Tonukari et al., 2000). Deletion of the *A. brassicicola SNF1* homolog did not significantly affect virulence, in contrast to the observations made for several other fungi (Ospina-Giraldo et al., 2003; Tonukari et al., 2000). Moreover, deletion of the *A. brassicicola Pac*C gene, which has been shown to be important for virulence in *S. sclerotiorum* (Rollins, 2003), did not affect virulence in this fungus, suggesting that pH response does not play an important role in pathogenesis of *A. brassicicola*. These results collectively demonstrate that every fungal necrotroph is distinct in that each employs a unique arsenal of weapons and armament in order to successfully cause disease.

Deletion of the Ste12 homolog, a transcription factor downstream of Amk1, resulted in loss of pathogenicity as was described in other pathogenic fungi (Xu, 2000). However, Δ*abste12* mutants were capable of infecting wounded plants. Also in this study, the ability of mutants with deletion of *Snf1*, *Ste12*, or *XlnR* (a transcription factor critical for xylanase production) to utilize complex carbon sources (xylan, cellulose, and pectin) was investigated (Cho et al., 2009). In these experiments, only mutants deleted for *XlnR* were unable to utilize a specific carbon source, in this case xylan. The *Ste12* deletion mutants were less pathogenic but could still utilize all carbon sources tested, suggesting that regulation of production of cell wall-degrading enzymes in *A. brassicicola* is under a different control regime from that of other fungi studied to date and that xylanase is not important for pathogenicity. All of the genes described above are single-copy genes in the *A. brassicicola* genome, and the likelihood of functional redundancy due to other closely related genes is considered low.

As part of the study by Cho et al. (2009), two novel virulence factors were discovered predicted to encode a transcription factor (*AbPro1*) and a two-component histidine kinase gene (*AbNIK1*). Deletion of *AbPro1* resulted in a 70% reduction in virulence, and this mutant also exhibited a 25% reduction in vegetative growth rates in vitro. Deletion of *AbNIK1* resulted in a near complete loss of virulence without changes in vegetative growth rates in vitro. Importantly, the addition of tryptone to spores of both Δ*abste12* and Δ*abnik1* during plant inoculation resulted in a complete restoration of pathogenicity. These results might suggest the presence of a previously undescribed nutrient- or polypeptide-sensing pathway downstream of Amk1/AbSte12 signaling pathways and a putative AbNIK1 osmoregulation pathway. Further investigation into this phenomenon is certainly warranted. In addition to *AbNIK1* and *Amk1*, the *A. brassicicola* Slt2 MAPK and HOG MAPK homologs have been knocked out and characterized (Lawrence, unpublished). Both of these kinases are pathogenicity factors in phytopathogenic fungi (for a review, see Xu, 2000). Slt2 has been shown to be associated with cell wall integrity and HOG with oxidative stress tolerance (Xu, 2000). The Slt2 homolog is a major virulence determinant in *A. brassicicola* (Y. Cho, D. C. Scott, and C. Lawrence, unpublished data). However, deletion of the HOG kinase homolog in *A. brassicicola* did not result in reduced virulence, suggesting that the fungus uses alternative mechanisms for oxidative stress tolerance (Lawrence, unpublished). Interestingly, deletion of a novel gene called TmpL (transmembrane protein L) in *A. brassicicola* has been shown to be required for virulence (Kim et al., 2009). Preliminary results strongly suggest that this protein is localized in Woronin body membranes and regulates levels of intracellular ROS. Compared to the wild type, Δ*atm1* mutants showed enhanced constitutive production of intracellular ROS, such as superoxide and hydrogen peroxide, upregulation of antioxidant genes (catalase, SOD, and glutathione peroxidase), enhanced sensitivity to external oxidative stress, and nuclear localization of the yeast oxidative stress-associated Yap1 transcription factor ortholog. The exact function of the Atm1 protein has yet to be determined.

In a recent study, disruption of *Aso-1*, a gene required for hyphal fusion (anastomosis), was also shown to be required for pathogenicity in *A. brassicicola* (Craven et al., 2008). In this study, data indicated that the *amk1* kinase mutants also failed to undergo hyphal fusion, suggesting a link between MAPK signaling and anastomosis.

In summary, considerable progress has been made over the last several years regarding identification of *A. brassicicola* virulence-associated genes. Well over 100 genes have been functionally analyzed through gene knockout and overexpression experiments, making *A. brassicicola* the species of choice for functional genomics research to define virulence mechanisms for this important genus of fungi (Cho et al., 2006, 2007, 2009; Craven et al., 2008; Kim et al., 2007; Oide et al., 2006).

CONCLUSION

The three fungal pathogens discussed in this chapter exhibit distinct but overlapping lifestyles and strategies for effective pathogenesis. In the current postgenomic era, these fungi are excellent models for studying necrotrophic pathogenesis, the development of sclerotia, sexual development, and host-pathogen compatibility via fungus-induced plant cell death. A combination of comparative genomics and functional genomics is currently ongoing on all these aspects and will undoubtedly yield fundamental information that can be exploited for improving disease control strategies to reduce the enormous economic damage that necrotrophic pathogens cause in a wide spectrum of crop species.

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Epichloë Endophytes: Models of an Ecological Strategy

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Endophytes, broadly defined, are organisms that colonize the internal tissues of plants. So many microorganisms "make their living" as endophytes, and this use of the term conveys so little information that it could be used interchangeably with the label "partner" or "symbiont." In common usage, the term endophyte usually refers to a bacterial or a fungal species that lives as an asymptomatic resident in the plant. It turns out that in nature most plants are teaming with endophytic partners (Rodriguez and Redman, 2008; Schardl and Wilkinson, 2000). Thus, it is important to understand the circumstances and currencies that induce fungi to employ this strategy. Rodriguez et al. (2008) recently divided fungal endophytes into two groups: clavicipitaceous and nonclavicipitaceous. Nonclavicipitaceous fungal endophytes are (i) prevalent among terrestrial plant lineages; (ii) phylogenetically diverse, spanning both the Ascomycota (most) and the Basidiomycota (fewer); and (iii) often poorly understood with respect to ecological roles. Clavicipitaceous endophytes are grass-associated fungi in the genera *Epichloë*, *Neotyphodium*, *Balansia*, *Myriogenospora*, *Atkinsonella*, *Parepichloë*, and *Aciculosporium* within the family Clavicipitaceae. Several features associated with the clavicipitaceous endophytes make them particularly good models for understanding this strategy: (i) systemic establishment occurs within the majority of the plant tissues; (ii) plant hosts serve as the sole source of nutrition for the majority of the fungal life cycle; (iii) many of these fungi produce secondary metabolites that act to enhance host fitness; (iv) fungal transmission is usually tightly linked to the host reproductive cycle; (v) the host ranges of each of the species are relatively narrow; and (vi) the relatedness of fungi in this group provides for excellent comparisons of the features among naturally occurring taxa. The term "epichloë endophytes" (plural, epichloae) (Table 1) is used to refer to species in the genera *Epichloë* (teleomorphs) and *Neotyphodium* (anamorphs). The unit of interaction for epichloae has been dubbed the symbiotum (plural, symbiota), which refers to a particular fungus-host combination. This chapter considers our current understanding of the epichloae (Fig. 1), for which the recently sequenced *Epichloë festucae* (www.endophyte.uky.edu) is considered a model (Schardl, 2001; Schardl et al., 2009).

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EVOLUTION OF EPICHLOAE

There are 11 known *Epichloë* species and 38 documented *Neotyphodium* species, of which 19 have been formally characterized and named (Table 1). The epichloae are in the family Clavicipitaceae within the order Hypocreales. Constituted by pathogens of animals, fungi, and plants as well as the epichloë symbionts, this family has unparalleled ecological diversity within its ranks. Spatafora et al. (2007) used multigene phylogenetic analysis and ancestral character state reconstruction to resolve the relationships among 54 clavicipitaceous taxa (Spatafora et al., 2007). Their work demonstrates that the epichloae are part of a derived clade containing *Epichloë* and *Neotyphodium* species and other plant-associated fungi (e.g., *Claviceps purpurea* and *Balansia henningsiana*). The presence of this group, nested within a large group of animal pathogens (e.g., *Metarhizum anisopliae*), indicates that leading up to the evolution of the clade there was an interkingdom host jump. Other examples of host jumps within this overall analysis tend to include jumps from pathogens of animals to pathogens of fungi (e.g., *Cordyceps fracta*), which occurred three to five times. This phylogenetic analysis also confirmed that the Clavicipitaceae are not monophyletic; rather, there are three distinct clades within the family (A, B, and C). Clavicipitaceae clade C includes fungi traditionally placed within the family (e.g., *Cordyceps cardinalis*) and also fungi from the family Hypocreaceae (e.g., *Hyphomyces plyporinus*). The genus *Cordyceps*, usually insect pathogens, has members in all three clades. Furthermore, each of the clades contains fungi with niches from two or three host kingdoms. Thus, glancing, as these authors did, at evolution of taxa within the

TABLE 1 Characterization of epichloae[a]

Species	Pedigree[b]	Host	Geographic origin	Transmission[c]	Alkaloid potential[d]
Epichloë spp.					
E. amarillans	Eam	*Agrostis* spp., *Sphenopholis* spp.	North America	Mixed	Ergot alkaloids, peramine
E. baconii	Eba	*Agrostis* spp., *Calamagrostis villosa*	Europe	Horizontal	Ergot alkaloids, peramine
E. brachyelytri	Ebe	*Brachyelytrum erectum*	North America	Mixed	Ergot alkaloids, peramine
E. bromicola	Ebr	*Bromus* spp., *Hordelymus europaeus*	Europe	Mixed/Vertical	Ergot alkaloids, **peramine**
E. clarkii	ETC	*Holcus lanatus*	Europe	Horizontal	Ergot alkaloids
E. elymi	Eel	*Elymus* spp., *Bromus kalmii*	North America	Mixed	**Peramine**
E. festucae	Efe	*Festuca* spp., *Lolium* spp.	Europe	Mixed	**Ergot alkaloids, lolines, indole-diterpenes, peramine**
E. glyceriae	Egl	*Glyceria striata*	North America	Horizontal	Ergot alkaloids, **lolines**
E. sylvatica	ETC	*Brachypodium sylvaticum*	Europe	Mixed	
E. typhina	ETC	*Anthoxanthum odoratum*, *Arrhenatherum elatius*, *Brachypodium pinnatum*, *Brachypodium phoenicoides*, *Lolium perenne*, *Phleum pratense*, *Poa* spp., *Puccinellia distans*	Europe	Horizontal	**Peramine**
E. yangzii	Eya	*Roegneria kamoji*	Asia	Mixed	
Neotyphodium spp.					
N. aotearoae	Nao	*Echinopogon ovatus*	New Zealand, Australia	Vertical	**Lolines**, indole-diterpenes
N. australiense	Efe, ETC	*E. ovatus*	Australia	Vertical	Ergot alkaloids, peramine
N. chisosum	Eam, Ebr, ETC	*Achnatherum eminens*	North America	Vertical	
N. coenophialum	Efe, ETC, LAE	*Lolium arundinaceum*	Europe, North Africa	Vertical	**Ergot alkaloids, lolines,** indole-diterpenes, **peramine**
N. funkii	Eel, Efe	*Achnatherum robustum*	North America	Vertical	**Ergot alkaloids, lolines,** indole-diterpenes
N. gansuense	Nin	*Achnatherum inebrians*	Europe	Vertical	
N. guerinii	ETC, Nin	*Melica ciliata*	Europe	Vertical	
N. huerfanum	ETC	*Festuca arizonica*	North America	Vertical	Peramine
N. inebrians	Nin	*A. inebrians*	Asia	Vertical	Ergot alkaloids
N. lolii	Efe	*L. perenne* subsp. *perenne*	Europe	Vertical	**Ergot alkaloids, indole-diterpenes, peramine**
N. melicicola	Efe, Nao	*Melica decumbens*	South Africa	Vertical	Indole-diterpenes, peramine
N. occultans	Ebr, LAE	*L. perenne* subspp. (annual)	Europe	Vertical	**Lolines**
N. pampeanum	Efe, ETC	*Bromus auleticus*	South America	Vertical	
N. siegelii	Ebr, Efe	*Lolium pratense*	Europe	Vertical	**Lolines,** indole-diterpenes, peramine
N. sinicum	Eya, ETC	*Roegneria* spp. (*Elymus* spp.)	Asia	Vertical	

(Continued on next page)

TABLE 1 (*Continued*)

Species	Pedigree[b]	Host	Geographic origin	Transmission[c]	Alkaloid potential[d]
N. stromatolongum		*Calamagrostis epigeios*	Asia	Vertical	
N. tembladerae	Efe, ETC	*Poa huecu*	South America	Vertical	Indole-diterpenes, **peramine**
N. tembladerae	Efe, ETC	*F. arizonica*	North America	Vertical	Indole-diterpenes, **peramine**
N. typhinum var. *canariense*	ETC	*Lolium edwardii*	Canary Islands	Vertical	
N. uncinatum	Ebr, ETC	*L. pratense*	Europe	Vertical	**Lolines**
Neotyphodium sp. AroTG-1	Eel, Efe	*A. robustum*	North America	Vertical	
Neotyphodium sp. BbeTG-1	Ebr	*Bromus benekenii*	South America	Vertical	
Neotyphodium sp. BpuTG-1	Eel	*Bromus purgans*	South America	Vertical	
Neotyphodium sp. EcaTG-1	Eam, Eel	*Elymus canadensis*	North America	Vertical	
Neotyphodium sp. EviTG-1	Eam	*Elymus virginicus*	North America	Vertical	
Neotyphodium sp. FaTG-2	Efe, LAE	*Lolium* sp.	South Europe, North Africa	Vertical	**Ergot alkaloids, indole-diterpenes**
Neotyphodium sp. FaTG-3	ETC, LAE	*Lolium* sp.	South Europe, North Africa	Vertical	**Lolines, peramine**
Neotyphodium sp. FalTG-1	Ebr, ETC	*Festuca altissima*	Europe	Vertical	
Neotyphodium sp. FobTG-1	Efe	*Festuca obtusa*	North America	Vertical	
Neotyphodium sp. FpaTG-1	Eam, ETC	*Festuca paradoxa*	North America	Vertical	
Neotyphodium sp. HeuTG-1	Ebr	*Hordelymus europaeus*	Europe	Vertical	
Neotyphodium sp. HeuTG-2	Ebr, ETC	*H. europaeus*	Europe	Vertical	
Neotyphodium sp. HboTG-1	Eam, Eel	*Hordeum bogdanii*	Asia	Vertical	
Neotyphodium sp. HboTG-2	Ebr, ETC	*H. bogdanii*	Asia	Vertical	Peramine
Neotyphodium sp. HbrTG-1	Ebr	*Hordeum brevisubulatum*	Asia	Vertical	
Neotyphodium sp. HbrTG-2	Ebr, ETC	*H. brevisubulatum*	Asia	Vertical	
Neotyphodium sp. LpTG-2	Efe, ETC	*L. perenne* subsp. *perenne*	Europe	Vertical	**Ergot alkaloids, indole-diterpenes, peramine**
Neotyphodium sp. PauTG-1	Eel, ETC	*Poa autumnalis*	North America	Vertical	
Neotyphodium sp. PsyTG-1	ETC	*Poa sylvestris*	North America	Vertical	

[a]Modified from Schardl and Leuchtmann, 2005; Moon et al., 2004; and Clay and Schardl, 2002; some data also from Moon et al., 2007; Ji et al., 2009; Yan et al. 2009; Li et al., 2006; and Iannone et al., 2009.

[b]Eam, *Epichloë amarillans*; Eba, *E. baconii*; Ebe, *E. brachyelytri*; Ebr, *E. bromicola*; Eel, *E. elymi*; Efe, *E. festucae*; Egl, *E. glyceriae*; Eya, *E. yangzii*; ETC, *E. typhina* complex; LAE, *Lolium*-associated clade; Nao, *Neotyphodium aotearoae*; Nin, *N. inebrians*.

[c]Vertical, transmitted only via asexual cycle; horizontal, transmitted only by sexual cycle; mixed, can be transmitted by either sexual or asexual cycle.

[d]Alkaloid potentials have been predicted based on the presence of the biosynthesis genes (Young et al., 2009; Johnson et al., 2007; Scott et al., 2009; Gonthier et al., 2008). Alkaloid production is dependent on the endophyte genotype, and each isolate may not be able to produce all alkaloids listed. Alkaloids which have been shown to be produced by an isolate are in boldface type. Not all species have been screened for alkaloid potential.

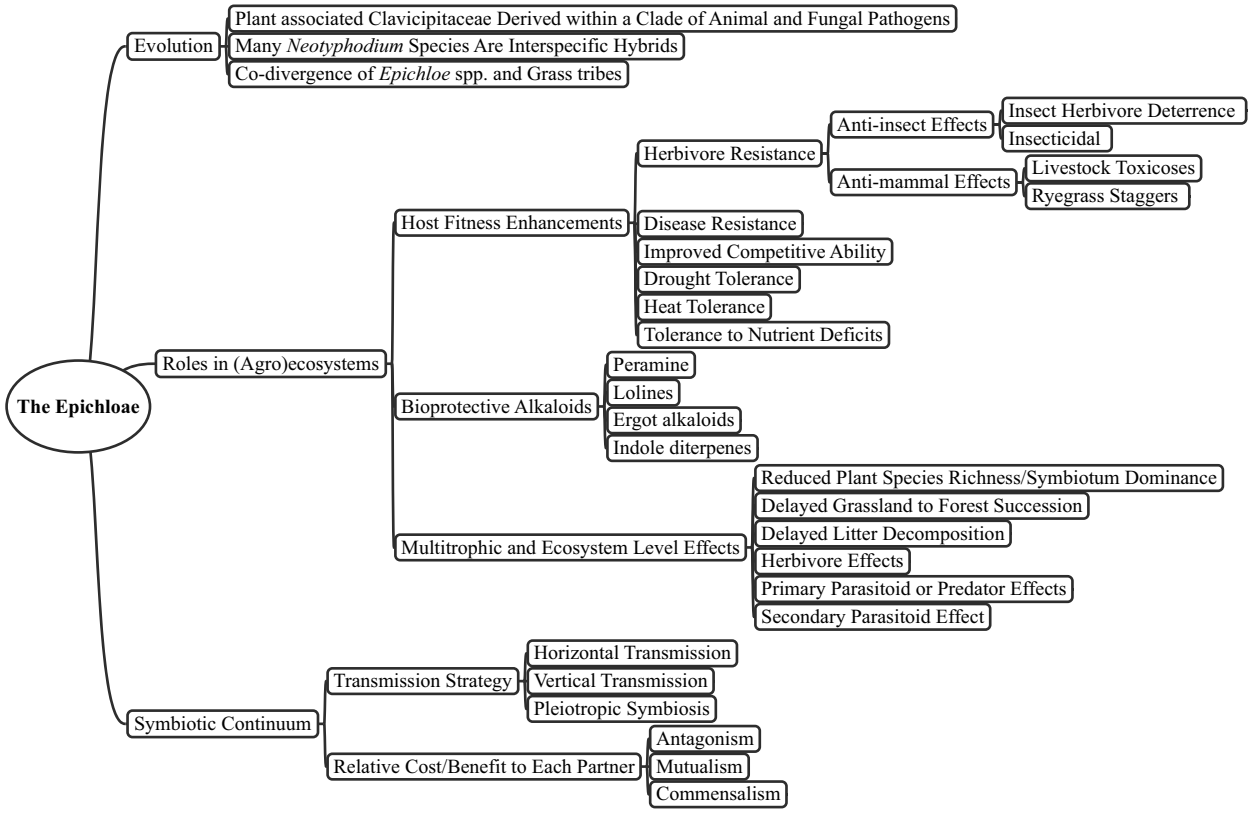

FIGURE 1 Important features of the epichloë endophyte ecological strategy. The evolution of this group of closely related fungi has been studied intensively, revealing a major host shift from animals to plants, interspecific hybridization as a prevalent feature for asexual species, and concomitant cladogenesis among these fungi and their grass hosts within the Pooideae. The agricultural and ecological roles of some endophytes have been especially well studied, thus providing a sense of the potential impacts of these symbiota at a variety of ecological scales. The frequently cited "symbiotic continuum" for the species within this group represents a combination of both types of transmission strategies and the costs and benefits for the host and endophyte in a given environment.

family Clavicipitaceae, host kingdom association was quite dynamic at that scale (Spatafora et al., 2007).

Evolution at a smaller scale within the epichloae provides a less dynamic picture of host affiliation; all *Epichloë* and *Neotyphodium* spp. are associated with grasses in the subfamily Pooideae. However, studies of the evolution of these taxa are particularly exciting because they have revealed both (i) the fascinating pattern of many interspecific hybridization events leading to most *Neotyphodium* species (see Moon et al., 2004) and (ii) a clear pattern of codivergence of the *Epichloë* spp. with host tribes (Craven et al., 2001; Schardl et al., 2008).

The discovery of more than one copy of the *tub2* gene in different isolates of the asexual endophytes associated with tall fescue led to the first discovery of interspecific hybridization in fungi (Tsai et al., 1994). Subsequently, this phenomenon has been identified in several fungal species (Schardl and Craven, 2003), allowing for tremendous evolutionary potential in the participating fungi. Interspecific hybridization is quite prevalent among the *Neotyphodium* spp. examined to date (Table 1): each of the hybrid species contains genes from two or three of the *Epichloë* spp. Moon et al. (2004) found that 63% of the 32 *Neotyphodium* species they examined were heteroploid. Schardl et al. (2009) argue that most evidence suggests that the interspecific hybridizations are due to hyphal fusion (made more likely because of a lack of heterokaryon incompatibility in *Epichloë* spp. [Chung and Schardl, 1997]) after a superinfection by two different endophyte species (see Fig. 15.2 in Schardl et al., 2009). The average genome size of the haploid sexual species and the interspecific hybrids has been estimated by quantitative Southern analysis and electrophoretic karyotypes (Kuldau et al., 1999; Murray et al., 1992). To date, the number of chromosomes has ranged from 4 to 13, with chromosomal DNA sizes from 0.4 to at least 10.8 Mb. The genome sizes of haploid species such as *Epichloë typhina*, *E. festucae*, and *Neotyphodium lolii* were estimated at ~28 to 35 Mb (Kuldau et al., 1999). As expected, interspecific hybrids have a larger genome, of up to ~57 Mb, consistent with the presence of multiple copies of genes. *Neotyphodium* sp. LpTG-2 (isolate Lp1), an interspecific hybrid of *E. festucae* and *E. typhina* progenitors, has a genome of ~55 Mb, whereas that of *Neotyphidium coenophialum*, with ancestors from the *E. festucae*, *E. typhina* complex (ETC), and the *Lolium*-associated endophyte (LAE) clade is ~57 Mb (Kuldau et al., 1999). The Lp1 genome size is almost the sum of the two ancestral genomes, but the *N. coenophialum* genome, consisting of three ancestors, is smaller than expected, presumably due to loss of some chromosomal material. This is supported by sequence

data wherein three alleles are detected for some but not all loci within *N. coenophialum* (Moon et al., 2004; Tsai et al., 1994). Thus, many of the *Neotyphodium* species have complex histories of interspecific hybridization, sorting, and gene loss, yielding less than a perfect complement of the genes from the progenitor *Epichloë* species.

The nonhybrid *Neotyphodium* species may result from *Epichloë* species that have lost the sexual cycle, or at least no expression of the sexual cycle has been observed in nature. Loss of sexual reproduction is common in fungi (for examples, see Gow, 2005, and Nielsen and Heitman, 2007). The lack of observation of the sexual stage in nature is probably even more common. Some experiments using cultures of *Neotyphodium* species as spermatia in crosses with stromata (female fruiting bodies) from the most closely related *Epichloë* species have revealed male fertility in some nonhybrid but not in hybrid *Neotyphodium* species (Moon et al., 2004; Brem and Leuchtmann, 2003).

Initially, the hypothesized codivergence of some *Epichloë* species with their host tribes was based on congruent shapes of the phylogenies of each species (Schardl and Wilkinson, 2000; Schardl et al., 1997; Wilkinson and Schardl, 1997). While these mirror phylogenies between symbiont and host are suggestive of codivergence, they do not necessarily mean that cladogenesis occurred simultaneously. More recently, Schardl et al. (2008) developed a more direct method to test for host-symbiont codivergence. By analyzing the correspondence of the most recent common ancestor ages at nodes in the trees of both the grasses and the endophytes, they were able to discern congruence in the timing of the divergences in partners. Thus, the codivergence indicates the ancient origin of epichloë endophytes in an early progenitor of the Pooideae (Schardl et al., 2008).

HOST FITNESS ENHANCEMENTS ARE WELL ESTABLISHED FOR AGRONOMICALLY IMPORTANT GRASSES

While it was not the first recognition of an epichloë endophyte, the discovery of an introduced tall fescue population by University of Kentucky agronomists in the early 1930s led to the development of the (in)famously popular cultivar Kentucky-31 (Stuedemann and Hoveland, 1988). Interest in the grass stemmed from its persistence and resilience in pastures and on hillsides during a time of recurring droughts in the region. The individuals in charge of the breeding program to develop the cultivar did not consider the effects of the endophyte *Neotyphodium coenophialum* because they were not aware that the seed-transmitted symbiont was present. Subsequent to its development, there was widespread employment of this cultivar for both forage and soil conservation purposes. By the late 1970s, it was established that a fungal endophyte in tall fescue was responsible for toxicity to livestock (Bacon et al., 1977). With this discovery and an improved understanding of the systemic, asymptomatic, and seed-transmissible qualities of the tall fescue endophyte, endophytes in other "toxic grasses" have been identified (reviewed by Schardl and Leuchtmann, 2005), not the least of which was *N. lolii* in perennial ryegrass in the southern hemisphere (Gallagher et al., 1984). Subsequent intensive study of the impact of these fungi on host fitness has revealed, ironically, that adopting these grasses for agriculture and conservation purposes is in large part due to the symbiosis that makes them toxic to livestock.

A flourish of studies comparing endophyte-infected (E+) and endophyte-free (E−) conspecific grasses in the 1980s and 1990s established a list of host fitness enhancements attributable to the endophyte (Fig. 1): herbivore resistance, disease resistance, competitive ability, drought tolerance, heat tolerance, and tolerance to nutrient deficits (reviewed by Schardl et al., 2009; Cheplick and Faeth, 2009; and Clay and Schardl, 2002). Perhaps not surprisingly, these studies focused on agriculturally important grasses. This likely means that those associations with the most pronounced effects were the ones that were studied because they were the ones selected for, and subsequently involved in cultivar development. Recent studies of some naturally occurring grasses have revealed that these sorts of pronounced effects are not necessarily the norm for all grass-epichloë endophyte associations (Faeth, 2002; Saikkonen et al., 2002, 2004). This represents an important area of consideration and an important rationale for treating all these species as components of one model for understanding endophytism. The variation in epichloë endophyte symbiota provides interesting opportunities to excavate the roles of particular traits, genotypes, life history strategies, and evolutionary histories in shaping interaction outcome.

Tall fescue is one of the most abundant forage and turf grasses used in the United States, spanning over 35 million acres (>14 million ha) (Ball et al., 1993; Thompson et al., 2001). Tall fescue is primarily grown in the Midwest, Southeast, and Pacific Northwest and is well adapted to the transition zone (also known as the fescue belt) that is considered colder than optimal for warm-season grasses but hotter than optimal for other cool-season grasses such as perennial ryegrass (Sleper and West, 1996). The *N. coenophialum* endophyte present in tall fescue has been extensively shown to produce ergot alkaloids that are detrimental to grazing animals, with an estimated annual loss to the beef cattle industry of $600 million (Hoveland, 1993). It is estimated that 80% of tall fescue acreage is highly infected with a toxic endophyte. Efforts to reseed with endophyte-free tall fescue, in order to provide toxin-free grass, result in pastures that are not persistent especially under biotic and abiotic stress (Arachevaleta et al., 1989). Similarly, it is also the case that perennial ryegrass predominantly grown in New Zealand and Australia was infected with *N. lolii*, an epichloë endophyte that produces indole-diterpenes, known as the causative agent of ryegrass staggers (Fletcher and Harvey, 1981; Gallagher et al., 1981, 1982; Prestidge et al., 1985a, 1985b). As was found with *N. coenophialum*, the presence of the endophyte in perennial ryegrass provided a greater advantage under insect pressure (Prestidge et al., 1985a, 1985b; Latch et al., 2000a; Popay et al., 1990). While the anti-insect alkaloids and tolerance to abiotic stress are highly desirable, the utility of these endophytes is limited due to the ergot alkaloids and lolitrems that are detrimental to grazing animals. With this in mind, the more toxic strains are now being replaced in tall fescue and perennial ryegrass by endophyte strains that do not produce these detrimental alkaloids (Latch et al., 2000a, 2000b; Bouton et al., 2002). Livestock grazing grasses infected with these "animal-friendly" strains showed greatly improved health and weight gains as much as twice that of animals grazing on the toxic strains while still providing the grass field persistence (Bouton et al., 2002; Bluett et al., 2005a; Bluett et al., 2005b; Hopkins and Alison, 2006; Nihsen et al., 2004; Parish et al., 2003a, 2003b; Watson et al., 2004). It is estimated that more than 200,000 acres of nontoxic endophyte-infected tall fescue has been planted in the United States (A. Hopkins, personal communication), while in New Zealand only nontoxic-endophyte-infected perennial ryegrass is marketed.

In contrast to the beneficial *N. coenophialum*, orchardgrass producers in the Willamette Valley, Oregon, have started seeing seed crops decimated by the choke pathogen, *Epichloë typhina*. The state of Oregon is the largest producer of tall fescue (158,000 acres) and orchardgrass (*Dactylis glomerata*; 16,000 acres) seed, with estimated values of $163 million and $13.5 million, respectively. Orchardgrass is primarily grown in the Northeast, parts of the Midwest (Iowa and Michigan), and to a lesser extent the Appalachian, Ozark, and Pacific Northwest regions, both in pastures and as mixtures with legumes for hay (Van Santen and Sleper, 1996). However, since 1998 there has been a significant increase in disease incidence: 60 to 90% of fields surveyed were found to be infested with the choke pathogen (Pfender and Alderman, 1999, 2006). It was estimated that the value of the loss of orchardgrass seed crops due to choke from *E. typhina* totaled $0.8 million (Pfender and Alderman, 2006).

MULTITROPHIC AND ECOSYSTEM LEVEL EFFECTS

Clay and Holah (1999) conducted a 4-year field study to determine and compare the impacts of E+ and E− tall fescue on a natural grass community. To do this, they plowed the existing plants and then seeded with E+ or E− tall fescue. Thus, as the stands reestablished, they consisted of the applied seeds and seeds in the soil seedbank from previous generations of forbs and grasses. The authors monitored the degree of endophyte infection in the plots to ensure they continued to represent the treatments of E+ versus E−. Through regular surveys over the 4-year period, they tracked both the species richness and the biomass accumulation in each treatment. There was significantly less plant species richness, due to tall fescue dominance, in the E+ stands. Overall the biomass accumulation was the same in the two treatments (Clay and Holah, 1999). Thus, they clearly demonstrated that the competitive advantage that was previously well established in short-term greenhouse and growth chamber studies translates into a dramatic impact on community composition over time. Furthermore, the implications of this study reveal the concern that when introduced symbiota with pronounced fitness enhancements become established in nature, they run the risk of dominating communities and driving out some native plant species.

In a subsequent study of the effect of E+ versus E− on the grassland-to-forest succession process, Clay and colleagues monitored the same site and an additional site seeded in a similar manner (Rudgers et al., 2007). The E+ stands exhibited suppression of succession, with substantially fewer, smaller trees and an altered tree composition, including the favoring of a nonnative species (white mulberry) at one of the sites. All the mechanisms by which the endophyte mediates reduced tree establishment and diversity were not necessarily elucidated in that one study. However, at the site where they examined rates of herbivory by voles (*Microtus* spp.) on tree seedlings, they clearly demonstrated that trees in the E+ plots experienced significantly higher losses. Suppressed succession on a large scale would clearly have important implications because the ecosystem services provided by both mature diverse grasslands and forests (e.g., carbon sequestration and wildlife habitat) would be at least delayed, if not lost.

One might expect that the pronounced impacts on species richness and succession are an artifact of "stacking" the comparison such that you have stands that are pure E+ versus E−. Clay et al. (2005) addressed this by seeding communities with equal parts E+ and E− tall fescue and then monitoring (i) the mammalian and insect herbivory, including treatments to exclude herbivores, and (ii) the relative abundance of each type of tall fescue and other plant species in each treatment. In plots open to herbivory by both insects and mammals, endophyte infection was 2.5 times greater than in plots where both herbivore types were excluded. Thus, in cases where stands might not be completely E+, impacts on herbivores likely manifest into shifts toward increasing the E+ proportion over time. Furthermore, in all treatments, whether herbivores were excluded or not, the proportion of endophyte infection increased over time (54 months total [Clay et al., 2005]). Thus, symbiota with pronounced fitness enhancements like tall fescue-*N. coenophialum* are likely to dominate plant communities where they occur, and this domination will only be accelerated when herbivore pressure is higher. These findings in a staged "natural" grass community are clearly consistent with findings in forage agroecosystems. Livestock grazing pressure certainly favors dominance of the E+ forage grasses (Jensen and Roulund, 2004). The differential herbivory might well be predictable given the antiherbivore alkaloid production established for tall fescue; however, large-scale and long-term studies of communities were important to understand how this would play out with respect to species richness, E+ dominance, and impacts on succession.

Other ecological interactions and ecological processes are also affected by these symbioses. Litter from E+ tall fescue decomposes more slowly, and the detritivore communities in E+ and in E− treatments were different (Lemons et al., 2005). Many studies have demonstrated the impacts of endophyte presence on predators of insect herbivores (reviewed by Faeth and Fagan, 2002; Hartley and Gange, 2009; and Omacini et al., 2001). Briefly, Finkes et al. (2006) found a significant reduction in spider species richness but not overall abundance in E+ tall fescue plots; however, the major mechanisms for this effect were not elucidated. In the perennial ryegrass-*N. lolii* system, ladybird beetles (*Coccinella septempunctata*) exhibited reduced reproductive success (reduced fecundity, developmental delays in larvae, and reduced survival) when they fed on aphids (*Rhopalosiphum padi*) reared on E+ grass (de Sassi et al., 2006). In the same system, Bultman et al. (2003) found differences among isolates with different alkaloid profiles in the impact on *Microctonus hyperodae*, a parasitoid of Argentine stem weevils. Isolates that expressed ergovaline had an impact on survival of the parasitoid, while the presence of any other alkaloid resulted in delayed parasitoid development. Harri et al. (2008) demonstrated a negative effect of E+ perennial ryegrass on a trophic level three rungs away in the food web, the secondary parasitoid *Asaphes vulgaris*. Clearly, in symbiota with pronounced host fitness enhancements, endophytes have great potential to affect communities on multiple trophic levels.

BIOPROTECTIVE ALKALOID PRODUCTION

Epichloë endophytes produce a range of bioactive secondary metabolites (see Fig. 2) including the ergot alkaloids (e.g., ergovaline), pyrrolizidines (e.g., lolines), a pyrrolopyrazine (e.g., peramine), and indole-diterpenes (e.g., lolitrem B). Synthesis of these four alkaloid groups is confined to endophyte-infected grasses. This observation together with several reports of the synthesis of these alkaloids in culture confirms that ergovaline, lolines, peramine, and lolitrems are of fungal origin (Blankenship et al., 2001; Penn and Mantle,

1994; Reinholz and Paul, 2001). Since the late 1970s, most studies have focused on understanding the chemistry behind the synthesis of these bioactive alkaloids based on the identification of compounds in endophyte-infected material, such as tillers and seeds. More recently, great advances have been made through the cloning and characterization of the biosynthesis genes required for alkaloid production (Damrongkool et al., 2005; Fleetwood et al., 2007; Panaccione et al., 2001; Spiering et al., 2002, 2005b; Tanaka et al., 2005; Wang et al., 2004; Young et al., 2005, 2006). In fungi, secondary metabolite genes commonly reside in functionally related gene clusters (Keller and Hohn, 1997). This is the case for the ergot alkaloid (*eas*), loline (*lol*), and lolitrem (*ltm*) biosynthetic genes (Fleetwood et al., 2007; Spiering et al., 2002, 2005b; Young et al., 2005, 2006).

Peramine and the lolines have anti-insect properties (Schardl and Wilkinson, 2000; Bush et al., 1997; Rowan and Latch, 1994). The ergot alkaloids and indole-diterpenoids are known to exhibit some protection against insects, but they are best known for their antimammalian biological activity. Of the endophytes screened to date (Table 1), many were able or have been predicted to produce peramine, some produced ergot alkaloids and lolines, and very few produced indole-diterpenes (Clay and Schardl, 2002; Christensen et al., 1993; Johnson et al., 2007; Siegel et al., 1990; Young et al., 2009; Scott et al., 2009). No single endophyte species produces all four alkaloid classes (Schardl et al., 2009); however, based on available genetic diversity within *E. festucae* it should be possible to breed an endophyte (for an example, see Wilkinson et al., 2000) that produces all four compounds.

The asexual species show a greater diversity of alkaloid profiles (Table 1), with many isolates able to produce more than one alkaloid, consistent with their expanded genomes. Generally speaking, the asexual species also produce the alkaloids in higher concentrations than their sexual relatives (Bush et al., 1997). However, alkaloid concentrations in planta can be influenced by seasonal conditions, host genotype, wounding tissue type, and age (Gonthier et al., 2008; Spiering et al., 2005a; Rowan and Latch, 1994; Siegel and Bush, 1997). The two best-studied symbiota (livestock toxicosis-associated) each produce three alkaloids (tall fescue-*N. coenophialum* produces lolines, peramine, and ergot alkaloids; and perennial ryegrass-*N. lolii* produces peramine, lolitrems, and ergot alkaloids). Clay and Schardl (2002) suggest that this pyramiding of alkaloid profiles has increased the fitness of the asexual species, thereby supporting a hypothesis that the hybrids are evolving under positive selection (Clay and Schardl, 2002).

Lolitrems (Fig. 2) are indole-diterpenes derived from the precursors indole-3-glycerol phosphate and geranylgeranyl diphosphate (reviewed by Saikia et al., 2008). Lolitrems are produced in planta, but small quantities of indole-diterpenes have been detected in plate cultures (Reinholz and Paul, 2001; Penn et al., 1993), thereby proving their fungal

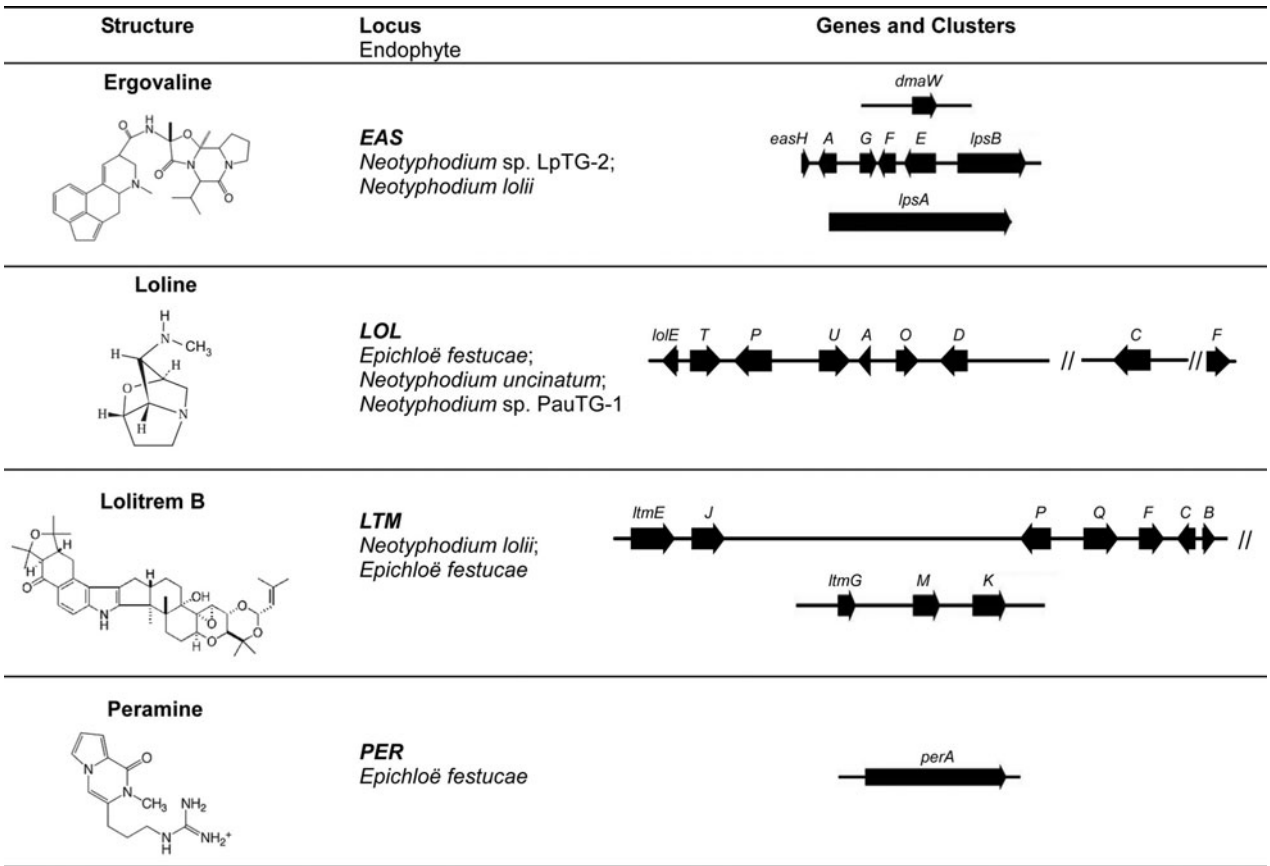

FIGURE 2 Bioprotective alkaloids and associated gene clusters of the epichloae. When possible, gene clusters from *E. festucae* or closely related asexual species are shown. Black arrows represent genes (drawn to scale), with the abbreviated gene name above. Repetitive elements are not shown. All clusters are drawn to scale with respect to each other. The following accession numbers were used: *EAS*, AY259837, AF368420, and EF125025; *LOL*, EF012265 and EF012267; *LTM*, DQ443465 and AY742903; *PER*, AB205145.

origin. Production of lolitrems is confined to *E. festucae* and those asexual derivatives of the *Neotyphodium* species that have an *E. festucae* progenitor (Young et al., 2009). Most *N. lolii* produce indole-diterpenes, but some naturally occurring isolates, such as AR1, have been identified that are devoid of the highly toxic lolitrems yet can produce simpler indole-diterpenes. Isolates such as AR1 still retain many of the desirable features of endophyte-infected grass without the associated toxicity and are now being deployed in perennial ryegrass as animal-friendly cultivars (Bluett et al., 2005a, 2005b; Fletcher, 1999; Tapper and Latch, 1999).

The indole-diterpene biosynthesis locus for lolitrem (*LTM*) contains 10 genes that form a complex gene cluster interspersed with repetitive elements (Young et al., 2005, 2006, 2009). The best-characterized indole-diterpene biosynthesis genes are the *pax* genes required for the production of paxilline, produced by the ascomycete *Penicillium paxilli* (McMillan et al., 2003; Saikia et al., 2006; Young et al., 2001). Precursors of paxilline, such as paspaline and 13-desoxypaxilline, have been identified in endophyte-infected grasses, which suggested they are likely intermediates in lolitrem production (Gallagher et al., 1984; Miles et al., 1994; Parker and Scott, 2004; Weedon and Mantle, 1987).

Sequence analysis at the *N. lolii LTM* locus revealed the presence of 10 genes, of which six predicted orthologs have been characterized in *P. paxilli* for the biosynthesis of paxilline (McMillan et al., 2003; Saikia et al., 2006, 2007; Young et al., 2001). The synthesis of the first identifiable intermediate, paspaline B, has been shown to require PaxG (geranylgeranyl diphosphate synthase), PaxM (monooxygenases), PaxC (prenyltransferase), and PaxB (unknown function) (Saikia et al., 2006), of which orthologous genes encoding LtmG, LtmM, LtmC, and LtmB are found at the *LTM* locus (Young et al., 2006). Heterologous expression systems have shown that the gene encoding PaxM will complement an *ltm*M gene disruption in the endophyte *E. festucae* (Young et al., 2005). Likewise, complementation of *P. paxilli* gene disruptions for *pax*M and *pax*C can be effected by expressing *ltm*M and *ltm*C under the control of the *pax*M promoter (Young et al., 2006).

The *LTM* locus contains four P450 monooxygenase genes (*ltmP*, *ltmQ*, *ltmJ*, and *ltmK*) of which two, *ltmP* and *ltmQ*, encode functional orthologs of *paxP* and *paxQ*, respectively. The remaining genes, *ltmJ*, *ltmK*, *ltmE* (multifunctional prenyltransferase), and *ltmF* (aromatic prenyltransferase), are predicted to encode enzymes that prenylate and modify the left-hand (LtmE and LtmJ) and right-hand (LtmF and LtmK) sides of the simpler indole-diterpene molecule (Young et al., 2009). These four genes appear to be unique to lolitrem biosynthesis and can function independently of one another. A metabolic grid rather than a linear pathway has been proposed for lolitrem biosynthesis based on isolation of multiple indole-diterpene compounds from *N. lolii*-infected perennial ryegrass (*Lolium perenne*) and analyses of naturally occurring isolates that have variation at the *LTM* locus (Young et al., 2009; Gatenby et al., 1999; Lane et al., 2000; Munday-Finch et al., 1995, 1998; reviewed by Schardl et al., 2009, and Scott et al., 2009). These data indicated that the left- and right-hand prenylations can occur in either order, thereby likely increasing the diversity of metabolites that can be produced by the fungus.

Using PCR to screen for the presence of the *ltm* genes has shown discontinuous distribution across the epichloae. However, the presence of core *ltm* genes correlates with the biosynthesis of indole-diterpene compounds (Young et al., 2009). A similar approach has been used to identify the alkaloid potential for ergot alkaloids, peramine, and lolines (Spiering et al., 2005b; Johnson et al., 2007; Scott et al., 2009) and provides a rapid screen to determine the alkaloid potential of an endophyte.

Approximately 50% of *Epichloë* and *Neotyphodium* species screened for ergot alkaloids (e.g., ergovaline) (Fig. 3) are able

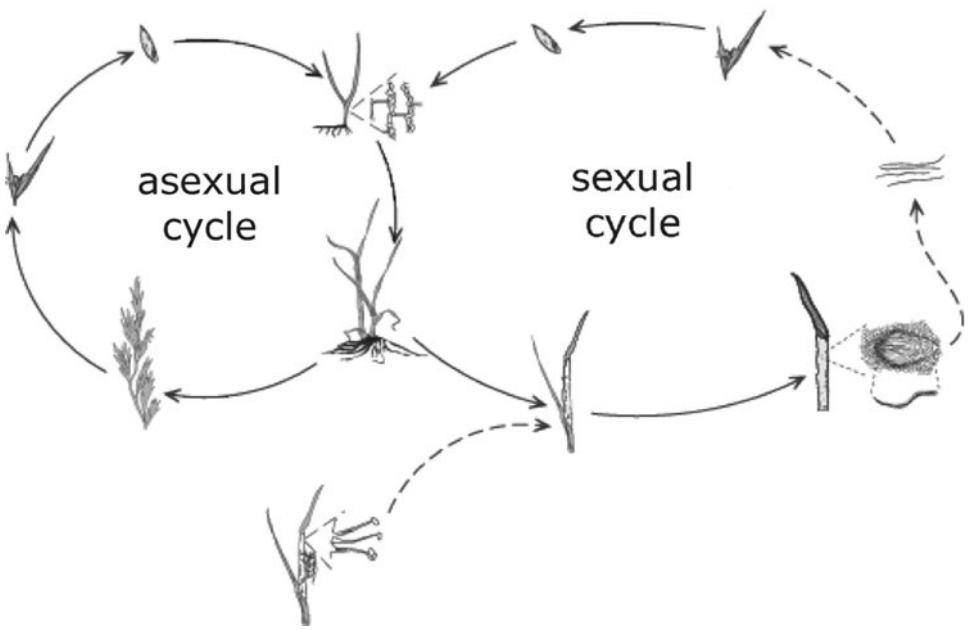

FIGURE 3 Life cycle diagram for the epichloae. The asexual *Neotyphodium* spp. exhibit only the cycle on the left (vertical transmission). Some of the sexual *Epichloë* spp. exhibit only the cycle illustrated on the right (horizontal transmission), while others, including *E. festucae*, exhibit the cycles shown on both sides of the figure on different tillers of the same plant (mixed transmission). Figure adapted from Clay and Schardl (2002).

to produce these compounds in planta (Siegel et al., 1990). These compounds provide some protection against insects (Siegel and Bush, 1997), but they are best known as the agents of livestock toxicosis. Genes involved in ergot alkaloid biosynthesis (*eas* genes) have been cloned from epichloë endophytes (Fleetwood et al., 2007; Panaccione et al., 2001; Wang et al., 2004), *Claviceps* spp. (also clavicipitaceous fungi that are pathogens of cereals) (Arntz and Tudzynski, 1997; Correia et al., 2003; Haarmann et al., 2005; Lorenz et al., 2007; Tsai et al., 1995; Tudzynski et al., 1999), and *Aspergillus fumigatus* (Coyle and Panaccione, 2005). As found with the *LTM* locus, the genes required for ergot alkaloid biosynthesis are contained as a complex gene cluster of at least 11 genes that are riddled with repetitive elements such as retrotransposons and miniature inverted-repeat transposable elements (Fleetwood, 2007; Fleetwood et al., 2007; Scott et al., 2009). While much of the understanding of ergot alkaloid biosynthesis has come from the characterization of the genes in the *Claviceps* spp., disruption and characterization of key pathway genes such as *dmaW*, *lpsA*, and *lpsB* have also been performed for epichloë endophytes (Fleetwood et al., 2007; Panaccione et al., 2001; Wang et al., 2004). A complication to studying ergot alkaloid biosynthesis in an endophyte such as *N. coenophialum* from tall fescue is the likely presence of at least two copies of each gene.

Lolines (Fig. 2) are pyrrolizidine alkaloids known for their potent anti-insect properties. The production of lolines can exceed 10,000 μg/g (dry weight), thus making them the most abundant alkaloid produced by endophytes (Bush et al., 1993). Approximately 35% of *Epichloë/Neotyphodium* species screened to date are able to produce lolines (Siegel et al., 1990; summarized by Clay and Schardl, 2002). Of the endophyte species tested, lolines have been identified in *N. aotearoae*, *N. occultans*, *N. uncinatum*, and in some *E. festucae* and *Neotyphodium* endophytes with an *E. festucae* ancestor.

Wilkinson et al. (Wilkinson et al., 2000) used Mendelian genetic analysis to screen for insect feeding in a $Lol^+ \times Lol^-$ *E. festucae* cross. Progeny segregated in a 1:1 ratio, supporting a single locus, designated *LOL*. Insect death cosegregated with loline production. In addition, an amplified fragment length polymorphism marker tightly linked to loline production was used for a subsequent map-based cloning approach in *E. festucae* (Kutil et al., 2007). In *N. uncinatum*, two transcripts, *lolA* and *lolC*, were identified as upregulated by suppression-subtractive hybridization (Spiering et al., 2002). It turns out the amplified fragment length polymorphism marker that strictly cosegregated with loline production mapped with *lolC* in that fungus. Eventually the identification and full description of the cluster of nine genes at the *LOL* locus involved a variety of genome walking techniques simultaneously in the genetic model *E. festucae* and the massive loline-producing *N. uncinatum* (Spiering et al., 2002; Kutil et al., 2007). The hybrid endophyte *N. uncinatum* has two copies of the locus, *LOL-1* and *LOL-2*. Phylogenetic analysis based on the concatenated coding sequences of nine *lol* genes from five clusters from four fungi, i.e., *E. festucae*, *N. uncinatum* (two clusters), *N. coenophialum*, and *Neotyphodium* sp. PauTG-1, was consistent with contribution of *LOL* loci from particular *Epichloë* progenitors to the hybrid species. In particular, *LOL-1* in *N. uncinatum* was likely derived from its *E. bromicola* parent, while *LOL-2* is very similar to copies of *LOL* from species with *E. typhina* as the contributor (see Fig. 3 in Kutil et al., 2007). Interestingly, while *LOL-2* and each of the clusters from the other *Neotyphodium* species in the study were extremely similar, suggesting the only common ancestor, *E. typhina*, as the source, no extant isolate of *E. typhina* has been identified as a producer of lolines (Schardl et al., 2007). The biochemical pathway has been painstakingly dissected in recent years (see Schardl et al., 2007, for a review).

Peramine (Fig. 2) acts as a feeding deterrent for *Listronotus bonariensis* (Argentine stem weevil) (Tanaka et al., 2005; Rowan and Latch, 1994). Most of the *Epichloë* and *Neotyphodium* endophytes screened to date are able to synthesize the alkaloid peramine (Table 1). The only *Epichloë* species that have not produced peramine are *E. baconii* and *E. clarkii* (Clay and Schardl, 2002). Peramine is a pyrrolopyrazine, which is proposed to be synthesized from proline and arginine (Lane et al., 2000). Recently, a single nonribosomal peptide synthetase has been cloned and characterized and shown by gene deletion to be involved in peramine production (Tanaka et al., 2005). The *perA* gene is a two-module nonribosomal peptide synthetase that is upregulated in planta.

In keeping with the concept of gene clusters for the synthesis of natural products (Keller and Hohn, 1997; reviewed by Keller et al., 2005), the genes involved in these seemingly dispensable pathways are found to be coregulated (Brown et al., 1996; Gardiner et al., 2004; Proctor et al., 2003). Regulation can in part be through regulators found within the cluster, such as for aflatoxin and trichothecene biosynthesis (Proctor et al., 1995; Woloshuk et al., 1994), but can also be due to global regulators involved via environmental cues. The known epichloë alkaloid biosynthesis gene clusters have all been shown to be coregulated and also highly upregulated in planta (Fleetwood et al., 2007; Panaccione et al., 2001; Spiering et al., 2002; Tanaka et al., 2005; Young et al., 2006). In fact, only *N. uncinatum* isolates are capable of producing an alkaloid (lolines) reproducibly and reliably in culture (Blankenship et al., 2001). Unlike many secondary metabolite clusters, none of the three gene clusters (*eas*, *ltm*, and *lol*) contain evidence of a pathway-specific regulatory gene.

SYMBIOTIC CONTINUUM

Epichloë endophyte-grass associations are all symbiotic in that they are intimate interspecific interactions wherein the symbiont requires the host for a significant portion of its life cycle. In this way, these endophytes exemplify the original meaning of the term symbiosis (DeBary, 1879), whereby there is no specific requirement for the outcome of the interaction between the partners (e.g., mutualism versus antagonism).

Epichloë endophytes are not found growing saprophytically, outside symbiota, in nature. All the fungal species live the vast majority of their life cycles (Fig. 3) within the host plant. Thus, these fungi derive their sustenance from nutrients within the host apoplast. Clearly, by virtue of serving as the niche for the fungus, in all cases of naturally occurring symbiota the host provides that benefit to the fungus. However, the degree to which each partner in a symbiotum incurs other costs or benefits is dependent on the fungal genotype, host genotype, and the environment (Cheplick and Faeth, 2009; Muller and Krauss, 2005). Not surprisingly, the specific genotypes within a symbiotum are responsible for qualitative nature of traits (e.g., absolute compatibility between partners; the presence or absence of bioprotective alkaloids; or the transmission strategy exhibited), while the environment is more likely to affect traits quantitatively (e.g., the percentage of endophyte-infected grasses in the population, the

amount of alkaloids produced, or the relative importance of a given transmission strategy in a season).

In addition to these considerations, calculations of the relative costs and benefits for partners in the symbiotum ought to also include the aspect of time. Community and ecosystem level effects of association may take multiple seasons to manifest, and thus, measurements of host fitness after just a single season might not capture these impacts. Time is also involved in rare catastrophic events that might strongly favor or even require the intact symbiosis for survival, such that populations that exhibit losses in percent endophyte infection over time due to small net costs will not survive if those losses are local extinctions prior to reoccurrence of the catastrophic event. However, if these events occur with enough regularity, endophyte infection might prevail without any measurable benefit (or even with some known costs) to the host in most years. Clearly, studies of these associations over short periods of time might not capture these more complex patterns of selection.

LIFE CYCLE

Endophytes in the genus *Neotyphodium* possess only an asexual life cycle (Fig. 3, left side), wherein a seed colonized by the endophyte germinates and grows into a mature systemically colonized plant that produces seed colonized by the endophyte. This transfer of an identical endophyte genotype from one plant to its progeny is vertical transmission. The pure vertical transmission strategy of the *Neotyphodium* endophytes places them under pronounced selection pressure to enhance host fitness. These endophytes are effectively trapped within that host lineage without any possibility of shifting hosts; thus, the most fit symbiota will persist and outcompete less fit symbiota or less fit uninfected grasses. In the absence of a fitness advantage, one might expect to see loss of the endophyte from the plant population because plants with a tendency to lose the endophyte at any stage would be favored.

Endophytes in the genus *Epichloë* possess the ability to express a sexual cycle (Fig. 3, right side). The sexual cycle includes the production of a stroma around the flag leaf sheath of a given tiller, such that the inflorescence for that tiller is choked and never emerges and no seeds are produced from that tiller. The stroma is made up of both female and male components. *Epichloë* spp. are obligate outcrossers (heterothallic). Natural cross-fertilization between opposite mating types involves a third symbiotic partner, an anthomyiid fly (*Botanophila* spp.) (Leuchtmann, 2007; Rao et al., 2005). The female flies feed on stromata throughout the population and lay their eggs in stromata as well. After oviposition the female fly defecates on the stroma, thereby inoculating it with an abundance of conidia, which serve as spermatia. At this point, the fly exhibits a fascinating dancing behavior that involves spreading the frass all over the stroma by using her ovipositor, resulting in fertilization of the entire fruiting body. As the fly progeny develop into larvae, they feed on some of the mature perithecia and ascospores. Despite these losses in fungal progeny, the association with the fly persists, most likely due to increased likelihood of mating relative to other less efficient means (e.g., by wind dispersal or neighboring stromata rubbing against each other). Stromata that contain fly eggs contain five to seven times higher numbers of fertilized perithecia (Bultman et al., 1995).

Some species in the genus *Epichloë* choke all the tillers of the host plant. This strategy of pure horizontal transmission results in selection pressures on the fungus very different from those of the pure vertical strategy. Since fungal ascospores released from a stroma must rapidly colonize the ovule of a neighboring plant (presumably a low-probability endeavor), it is reasonable to expect that there is strong selection on these fungi to have a broad host range and perhaps more-aggressive invasive growth. An important distinction to make about the idea of horizontal transmission in endophytes is that, unlike the idea of horizontal transmission in most parasites, in which there is not necessarily a change in the microbe genotype, a requisite for horizontal transmission of the endophyte is outcrossing. Presumably, this also contributes to the evolutionary potential for traits favored by a horizontal strategy in these species.

Some species in the genus *Epichloë* exhibit a "balanced" or "pleiotropic" symbiosis (Michalakis et al., 1992), such that some tillers of the host are choked (horizontal transmission) and some tillers produce endophyte-colonized seed (vertical transmission) (Fig. 3). Presumably, in these pleiotropic symbioses selection acts to refine the balance between these two modes of transmission, perhaps resulting in improved adaptive response to environmental conditions that favor either strategy. Clearly, identification of the developmental switch that gets thrown in order to commit to one or the other strategy in a given tiller is an important goal.

DISSECTING EPICHLOË GENOMES

A strain of *E. festucae* was chosen as sequence strain for the first epichloë endophyte genome project (www.endophyte.uky.edu). *E. festucae* is an excellent model for the group (Schardl, 2001), based on its relative advantages in genetic tractability including (i) Mendelian analysis (Wilkinson et al., 2000); (ii) a nonhybrid genome (29 Mb) (Kuldau et al., 1999); (iii) ease of transformation and gene knockout manipulations (Fleetwood et al., 2007; Tanaka et al., 2005; Young et al., 2005; May et al., 2008; Bryant et al., 2007); (iv) ease of culture with the more rapid growth rate associated with the *Epichloë* sexual species; (v) shared heredity with the two agriculturally important endophyte genomes, the nonhybrid but slow-growing *N. lolii* (Zhang et al., 2006) and the hybrid *N. coenophialum* (Tsai et al., 1994) (Table 1); and (vi) expression of both sexual and asexual reproduction cycles (Fig. 3) due to its pleiotropic strategy (Leuchtmann et al., 1994). The DNA sequence is at 20× coverage and is also populated with close to 10,000 expressed sequence tags generated to assist in the prediction of the 11,035 gene models (Schardl et al., 2009). The fact that *E. festucae* is closely related to many of the recently sequenced filamentous fungal genomes (e.g., *Fusarium*, *Trichoderma*, and *Neurospora*) adds an excellent opportunity to examine the roles of orthologous genes and gene networks across fungi with distinct ecological strategies.

By way of example, Scott and colleagues have demonstrated that several genes associated with regulation of reactive oxygen species contribute to the regulated growth associated with epichloë symbiota (Takemoto et al., 2006, 2007; Tanaka et al., 2006, 2008; Scott and Eaton, 2008). All epichloae predominantly grow between the intercellular spaces of the grass. *Epichloë* spp. have limited epiphytic growth associated with production of the fruiting body. To keep pace with its host, the endophyte is able to grow and divide by intercalary hyphal extension in the expansion zone of grass leaves, a rarely documented characteristic for a filamentous fungus (Christensen et al., 2002, 2008). Endophytic growth is highly regulated in that it not only is synchronized to track host growth but also, under normal

conditions, does not evoke any host defense responses. Disruption of a variety of genes associated with the production of reactive oxygen species NoxA, NoxR, and RacA results in a hyperbranching pattern of (unregulated) fungal growth and stunting of the host plant (Takemoto et al., 2006, 2007; Tanaka et al., 2006, 2008). Thus, one model for the fine-tuned regulation of endophytic growth in planta is fungal mediation of reactive oxygen species. These findings are consistent with the roles of reactive oxygen species in regulation of cell differentiation and development in other fungi, based on disruption of orthologous genes (Aguirre et al., 2005; Scott and Eaton, 2008).

Until the advent of the *E. festucae* genome sequence, the repetitive elements identified within the epichloae had resulted from their presence within the sequenced gene clusters (Fleetwood et al., 2007; Young et al., 2005, 2006, 2009). Many fungal genomes are known to contain repetitive elements such as the transposable elements but vary in the numbers of these repetitive sequences (Cuomo et al., 2007; Daboussi, 1996; Daboussi and Capy, 2003; Dean et al., 2005; Galagan et al., 2003, 2005; Hane et al., 2007; Martin et al., 2008). For example, *Neurospora crassa* and *Fusarium graminearum* (Cuomo et al., 2007; Galagan et al., 2003) are relatively devoid of repetitive elements in comparison to the basidiomycete *Laccaria bicolor* (Martin et al., 2008). Sequence analyses of the *EAS*, *LTM*, and *LOL* gene clusters from isolates that contain *E. festucae* genomes have shown that they are riddled with retrotransposons, miniature inverted-repeat transposable elements, and AT-rich highly repetitive sequences (Fleetwood et al., 2007; Kutil et al. 2007; Young et al., 2005, 2006, 2009).

The epichloë genomes harbor many repeated sequences that range in copy number from low (<10) to high (>50) (Young et al., 2005, 2009). The first well-characterized repetitive elements, the degenerate retrotransposons Tahi and Rua, were identified as AT-rich regions present at the *LTM* locus of *N. lolii*. Subsequently, partial sequences of these elements were identified within the *EAS* locus, adjacent to *lps*A and within the *LOL* locus from *E. festucae*. Hybridization with Tahi and Rua showed that only the *E. festucae* and *E. baconii* or asexual species that have an *E. festucae* progenitor have detectible elements in their genomes (Young et al., 2005, 2009). While the retrotransposons have been found in the secondary metabolite gene clusters of *E. festucae*, these elements have not appeared in the alkaloid gene clusters that have originated from the other *Epichloë* spp. such as the *LOL* loci from *N. uncinatum*. The Rua long-terminal repeat associated with the *LTM* locus is also found with the *E. festucae LOL* locus but was not identified within the homologous locus from *N. uncinatum* (a hybrid of *E. typhina* and *E. bromicola*) or the *Neotyphodium* sp. PauTG-1 (a hybrid of *E. typhina* and *E. elymi*) (C. Young, unpublished data). An extensive analysis of the *EAS* gene cluster revealed that there were at least 10 different transposable elements within the 19.3-kb sequence (Fleetwood et al., 2007; Fleetwood, 2007). Many of these elements have now been identified within the *EAS*, *LOL*, and *LTM* gene clusters (Young et al., 2009; Fleetwood, 2007; Kutil et al., 2007). Analysis of the *E. festucae* genome sequence using BLAST with characterized long-terminal-repeat sequences indicates that there are between 30 and 100 independent insertions of each examined element and these sequences are typically found at the end of contigs and on contigs of <2 kb (Young, unpublished).

To date, all the retrotransposon sequences identified within *Epichloë* and *Neotyphodium* spp. lack open reading frames and contain a sequence bias towards A and T nucleotides. The high AT content of the repeated sequences suggested that a repeat-induced point mutation (RIP)-like mechanism (see chapter 10 of this book) may be functional within *Epichloë* species (Young et al., 2005, 2006; Fleetwood, 2007). RIP is a process that functions premeiotically and specifically and extensively mutates repetitive DNA by introducing C-to-T transitions (Galagan and Selker, 2004; Selker, 1990). The availability of genome sequences has revealed the likely evidence of RIP in many fungi (Cuomo et al., 2007; Dean et al., 2005; Galagan et al., 2003, 2005; Hane et al., 2007), of which few have been experimentally confirmed (Cuomo et al., 2007; Idnurm and Howlett, 2003). It is highly unlikely that RIP still occurs within the asexual species; therefore, most transitions in these repeats probably occurred prior to the loss of the sexual state. This is supported by identical partially "RIP'd" (i.e., subjected to RIP) Rua retrotransposon sequences located adjacent to *ltm*G in the sexual *E. festucae* strain Fl1 and in the asexual *N. lolii* strain Lp19 (Young et al., 2005).

The cloning of the known alkaloid biosynthesis genes (encoding ergot alkaloids, lolines, indole-diterpenes, and peramine) preceded the availability of the *E. festucae* genome sequence but has subsequently been beneficial for comparative analyses. Of great interest will be the location of the secondary metabolite gene clusters to determine if these will be found in subtelomeric regions. The genome sequence now provides a resource to look for other as-yet-unknown metabolite biosynthesis genes that are often made evident by the presence of certain classes of enzymes such as polyketide synthases, nonribosomal peptide synthetases, and prenyltransferases. Recently a web-based software prediction tool based on Secondary Metabolite Unique Regions Finder (SMURF) has become available (www.jcvi.org/smurf/) to help identify secondary metabolite gene clusters.

While we have now made major advances to understand the association of the endophyte in its grass host, we still have a long way to go before we understand both interacting parties. To date, the endophyte genome sequencing far exceeds the sequence data available for the agronomic grasses of interest. However, *Brachypodium distachyon* has recently been selected as a model grass for feedstock plants such as wheat and switchgrass (http://www.jgi.doe.gov/sequencing/why/CSP2007/brachypodium.html), and the genome sequence is available through the Joint Genome Institutes (http://www.brachypodium.org/). *Epichloë* endophytes in *Brachypodium* spp. have been well documented (Brem and Leuchtmann, 1999; Leuchtmann and Clay, 1993; Leuchtmann and Schardl, 1998), and therefore, the *B. distachyon* genome sequence and resources could be of considerable interest to the epichloë endophyte community as a model host to study endophyte-grass interactions.

For some model filamentous fungi the development of a genetic toolbox has been relatively uncomplicated because the organism is particularly amenable to manipulation (e.g., *N. crassa* and *Aspergillus nidulans*). Application of reverse genetics approaches in these systems has led to the elucidation of a variety of interesting phenotypes, for which an ecological role may or may not be hypothesized (e.g., identification of the role of sterigmatocystin in *A. nidulans* fitness [Wilkinson et al., 2004]). In contrast, the epichloë endophytes have a variety of very well studied traits associated with this ecological strategy, and as with many systems benefiting from advances in genome research, within an extremely short time a wealth of genetic resources have become available to the community. Thus, we predict that our

understanding of the mechanistic basis for the key components of this strategy (Fig. 1) will likely continue to expand even more rapidly in the near future.

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Mycoparasitism

ADA VITERBO AND BENJAMIN A. HORWITZ

MYCOPARASITISM AND BIOCONTROL

Biocontrol in Modern Agriculture

Plant diseases play a significant role in the destruction of natural resources in agriculture. In particular, soilborne pathogens cause important losses, fungi being the most aggressive. As such, modern agriculture is highly dependent on the use of chemical pesticides to control plant pathogens. Growing awareness of the environmental damage caused by the use of chemical substances for plant disease control in agriculture and the increasing costs of petroleum-based pesticides have raised the need to study biological alternatives. Biological control, the use of specific organisms that interfere with plant pathogens and pests, is a nature-friendly, ecological approach to overcome the problems caused by standard chemical methods of plant protection. Biological control can be defined as "the action of parasites, predators, or pathogens in maintaining another organism's population density at a lower average than would occur in their absence" (DeBach and Rosen, 1991). The integration of biological with chemical control is even more promising, because of a possible synergism of effects. Biological control may be achieved by both direct and indirect strategies (Chet, 1987, 1993). The direct approach involves the introduction of specific microbial antagonists into soil or plant material (Cook and Baker, 1983). Indirect strategies include (i) the use of organic soil amendments that enhance the activity of indigenous microbial antagonists against a specific pathogen, and (ii) cross-protection, which is the induction of plant self-defense mechanisms against a particular pathogen by prior inoculation of the plant with a nonvirulent strain or other nonpathogenic rhizocompetent bacteria or fungi.

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Antagonistic Interactions

Antagonistic interactions among microorganisms in nature include antibiosis, competition, and parasitism (Fig. 1). Fungal biocontrol agents produce inhibitory metabolites (Howell, 1998). Different volatile and nonvolatile secondary metabolites have been characterized from several biocontrol agents by antibiotic assay-guided isolation, and the production of antibiotic molecules by certain strains is often well correlated with their biocontrol ability. These metabolites can inhibit fungal growth or spore germination (Fig. 1A). Antibiosis occurring during the saprophytic and antagonistic growth could act in concert with other mechanisms such as cell wall-degrading enzymes (Lorito et al., 1996; Schirmbock et al., 1994). Although *Trichoderma* species produce a wide range of antibiotics (Reino et al., 2008), little is known about the genes involved in their synthesis and only recently have some efforts been made in this direction (Carpenter et al., 2008; Mukherjee et al., 2006; Wiest et al., 2002). In most, if not all, of the filamentous fungi studied so far, the genes for secondary metabolism are in clusters of various lengths. Identification of these genes provides a first insight into the genetic basis of secondary metabolism. Nonribosomal peptide synthases (NRPS) (Marahiel, 1997) generate a diverse array of compounds, and the 18-module NRPS from *Trichoderma virens* was cloned; mutation of this gene eliminated production of this class of peptaibols (the name refers to peptides containing the nonprotein amino acid α-aminoisobutyric acid, aib) (Wiest et al., 2002). NRPS genes are being cloned from more *Trichoderma* species (Vizcaino et al., 2005). In addition to their likely role in antagonism against fungal hosts, the 18-mer peptaibols of *T. virens* elicit plant defense responses (Viterbo et al., 2007b).

The close proximity and cell-cell interaction during mycoparasitism imply that secondary metabolites could be delivered very efficiently from parasite to host under these conditions, although contact with the host is certainly not a prerequisite for antibiosis. A gene encoding a monooxygenase predicted to have a flavin cofactor was isolated in a screen for genes expressed by *Trichoderma hamatum* during

interaction with *Sclerotinia sclerotiorium* (Carpenter et al., 2008). Induction occurred only in contact with the host fungus and in a host-dependent manner (several soilborne pathogens but not *Botrytis cinerea*). The substrate of this monooxygenase remains to be identified.

Many plant pathogens require exogenous nutrients to successfully germinate, penetrate, and infect host tissue. Therefore, competition for limiting nutritional factors—mainly carbon, nitrogen, and iron—may result in biological control of plant pathogens. Finally, one fungus can directly attack another, and mycoparasitism is considered a major contributor to fungus-fungus antagonism. In this chapter mycoparasitism is presented as a fungus-fungus interaction. Mycoparasitism is defined as a direct attack on a fungal thallus, followed by nutrient utilization by the parasite. The hyphae of mycoparasites have been shown to attach to the host hyphae, and in some interactions they coil around the host (Fig. 1B). Barnett and Binder (Barnett and Binder, 1973) divided mycoparasitism into (i) necrotrophic parasitism, in which the relationships result in death of the host thallus, and (ii) biotrophic parasitism, in which the development of the parasite is favored by a living rather than a dead host structure. The antagonistic activity of necrotrophic mycoparasites is attributed to the production of antibiotics, toxins, and hydrolytic enzymes. Biotrophic mycoparasites, on the other hand, tend to have a more restricted host range and produce specialized structures (Fig. 1D) to absorb nutrients from their host (Manocha, 1990). Some examples of biotrophic and necrotrophic mycoparasites have been recently reviewed in detail (Viterbo et al., 2007a). These and additional examples are summarized in Table 1. The necrotrophs, primarily *Trichoderma* species, have a wider host range and less-specific mode of action, and perhaps for this reason more field and greenhouse trials have made use of these. Thus, *Trichoderma* species have also been the most-studied models. The genome of *Trichoderma reesei* (*Hypocrea jecorina*) was sequenced (Martinez et al., 2008). *T. reesei* is best known as a cellulose degrader and producer of enzymes, though a recent study has addressed its potential to antagonize *Pythium* (Seidl et al., 2006). Annotation of the genome sequences of two mycoparasites of soilborne plant pathogens, *Trichoderma atroviride* and *T. virens* (previously *Gliocladium virens*) is currently (2009) in progress (Joint Genome Institute, U.S. Department of Energy). *Trichoderma* species are overrepresented in this chapter, because they have been the focus of the most work at the molecular and cellular levels. It will be important though, not to neglect the biotrophs, which may provide specific antagonists against particular pathogen species. Furthermore, the ecology of epiphytic mycoparasites has received very little attention compared with soilborne biocontrol candidates (Andrews, 1992). There are relatively few biocontrol agents of epiphytic pathogens. Epiphytic isolates of *Clonostachys* and *Trichoderma* species are still the most important filamentous fungi for biocontrol in aerial plant parts together with *Fusarium* spp. and *Penicillium* spp. (Hoopen et al., 2003). The great variety of tissues and physiological development stages that are to be protected against infection and fluctuation of environmental conditions on aerial surfaces of field-grown plants are the main reasons for the restricted use of epiphytic biocontrol agents in greenhouse applications. Also in this case, epiphytic biocontrol agents have to be applied frequently or in formulations with nutritious or protective additives (Hoopen et al., 2003).

MYCOPARASITIC DEVELOPMENT: COILING AND OTHER INFECTION-RELATED STRUCTURES

The interaction of *Trichoderma* with soilborne pathogenic fungi is an excellent example of necrotrophic mycoparasitism. Mycoparasitism appears to be a complex process and depends on a combination of mechanisms, acting sequentially or together. *Trichoderma* is attracted to and grows towards its host, probably by chemotropism. Host signals may act at a distance without physical contact, and some of these signals may be released from the host by the action of the cell wall-degrading enzymes made by the mycoparasite (Cortes et al., 1998; Vinale et al., 2008; Zeilinger et al., 1999). Upon contact with the host, *Trichoderma* coils around or grows along the host hyphae (Fig. 1B) and forms hook-like structures (presumably appressoria) that aid in penetrating the host's cell wall (Elad et al., 1983b). Penetration thus seems to occur both mechanically (through appressorium or infection of cushion structures, from which a thin penetrating hypha is generated) and enzymatically, by secretion of an array of cell wall-degrading enzymes (Viterbo et al., 2002). Degradation of the cell wall was observed as holes (visible by scanning electron microscopy [SEM]) remaining where the mycoparasite had been attached (Fig. 2). The coiling phenomenon and appressorium formation have been reported for other mycoparasites as well (Tu, 1984). In *Trichoderma*, this reaction has been found to be rather specific, and lectin-carbohydrate interactions were proposed to mediate the attachment and recognition between *Trichoderma* and soilborne plant pathogenic fungi. Although coiling around the host is a visually striking interaction (Fig. 1), it is not clear to what extent coiling and attachment are actually required for antagonism. Mutants in a mitogen-activated protein kinase (MAPK) gene of *T. atroviride*, for example, increased coiling while weakening the ability to overgrow host fungi in plate confrontation assays. At the same time, other biocontrol-related attributes were enhanced (Reithner et al., 2007).

The biochemical correlates of these structural features of the mycoparasitic interaction are just beginning to be understood. Perhaps the first evidence for specific cell surface signaling came from the experiments of Elad et al. (1983a), who found a lectin activity biochemically. O (but not A and B) erythrocytes attached to hyphae of *Rhizoctonia solani* but not to its mycoparasite. Attachment was blocked by galactose, present in *Trichoderma harzianum* cell walls, and by fucose. Following up on this information and in an attempt to definitively isolate the active component, Inbar and Chet (1992, 1994) showed that lectin-coated nylon fibers could at least partially mimic the host, inducing coiling as well as CHIT102 expression. However, coiling could be induced by a number of lectins that are not directly related to those present on fungal hosts (Inbar and Chet, 1992; Rocha-Ramirez et al., 2002). Lectins have been implicated in another mycoparasitic system (in this case, causing disease of mushrooms, rather than the more usual biocontrol of pathogens): an *Agaricus bisporus* fruit body lectin bound to the *Verticillium fungicola* cell wall glucogalactomannan (Bernardo et al., 2004).

Benhamou and Chet (1993) observed that the interaction between *T. harzianum* and *R. solani* is mediated by a fine

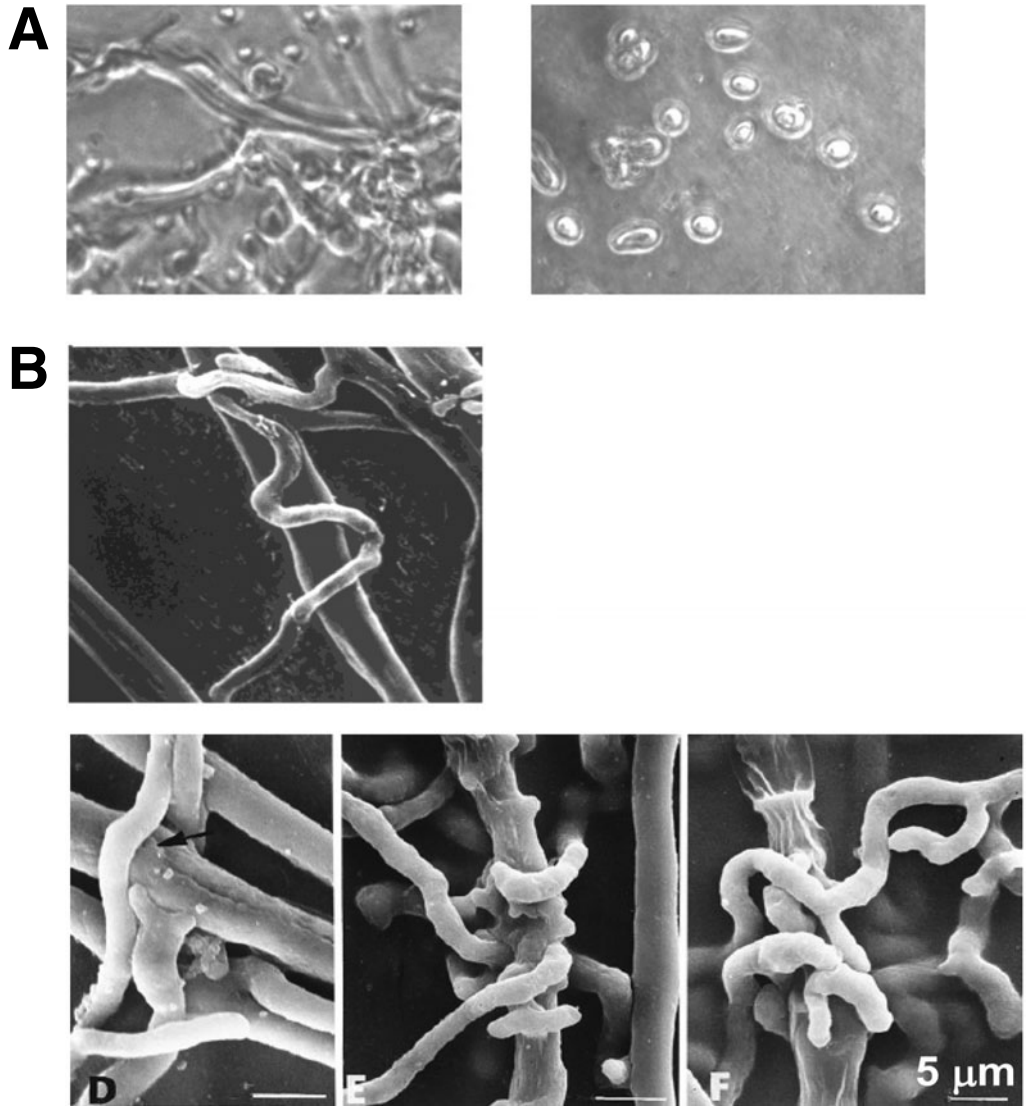
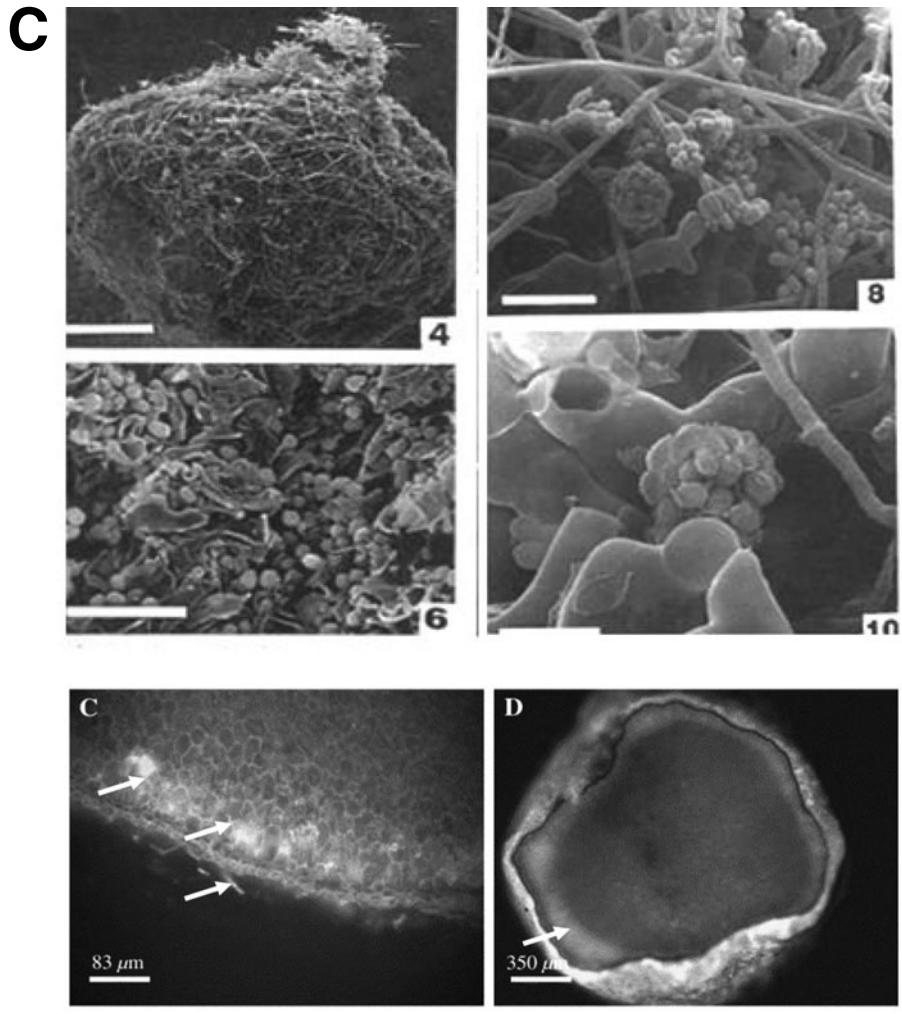


FIGURE 1 Basic mechanisms of antagonistic fungus-fungus interactions. (A) Antibiosis: culture filtrate from *Trichoderma* inhibits germination of *Botrytis* conidia. Shown are culture filtrates from *Trichoderma* grown on 2% glucose (left) and on 0.5% colloidal chitin (right). Growth of *Trichoderma* on chitin induces production of soluble antagonistic factors, which could be enzymes or small metabolites. (Reprinted from Viterbo et al., 2001, with permission of Blackwell Publishing Ltd.) (B) Necrotrophic mycoparasitic interactions: coiling of the mycoparasite around the host, shown here, is followed by destruction of the host. Upper panel, *Trichoderma asperellum* on *R. solani* (from Harman et al., 2004); lower panel, *P. oligandrum* hyphae interacting with *F. oxysporum* f. sp. *radicis-lycopersici*. (Reprinted with permission from Benhamou et al., 1997.) (C) Parasitism of sclerotia: an important interaction for biocontrol of soilborne disease. Upper four panels, SEM images (reprinted from Mukherjee et al., 1995b, with permission of Blackwell Publishing Ltd.). Lower two panels, fluorescence of GFP-expressing *Trichoderma* mycelia; fluorescent mycelia are indicated by arrows (reprinted from Sarrocco et al., 2006, with permission from Elsevier). (D) A biotrophic interaction: the parasite forms haustoria within the host cells, as in biotrophic fungus-plant interactions (Van Den Boogert and Deacon, 1994). Image reprinted from Deacon (2005) (Fig. 12-1), courtesy of Jim Deacon, The University of Edinburgh.



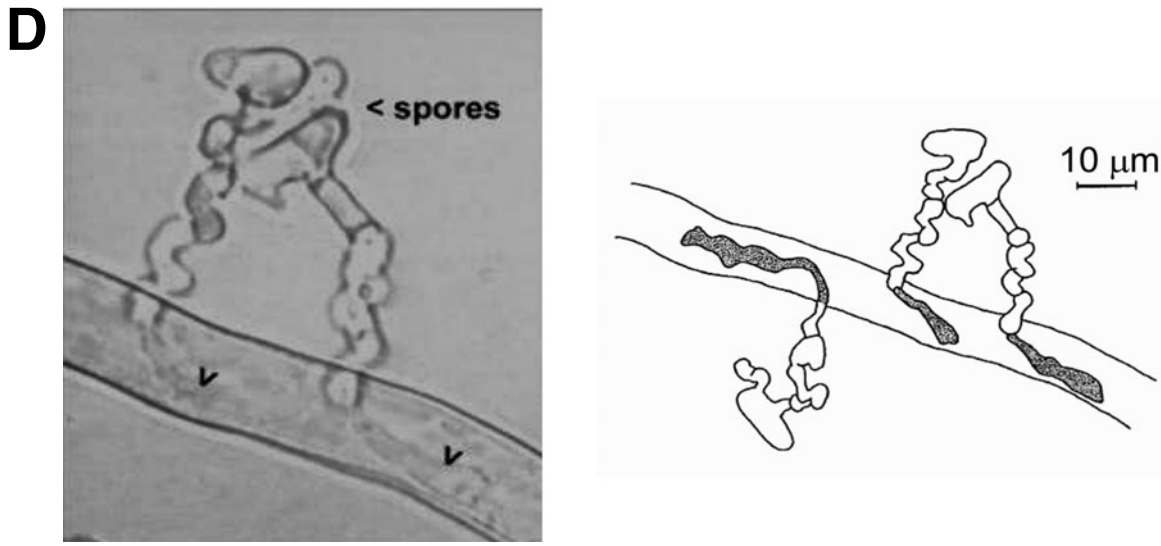


FIGURE 1 *(Continued)*.

TABLE 1 Summary of some mycoparasitic fungi applied in biocontrol or with potential as biocontrol agents

Species	Mode of interaction	Examples of host fungi	Reference(s)
S. sclerotivorum	Biotroph	Sclerotial fungi: *Sclerotinia sclerotiorium*, *Botrytis cinerea*	Del Rio et al., 2002; Fravel et al., 2002; Barnett and Ayers, 1981; Viterbo et al., 2007a
Ampelomyces quisqualis	Biotroph	Powdery mildews *Oidium*, *Erysiphe*	Abo-Foul et al., 1996; Viterbo et al., 2007a; Falk et al., 1995
Phoma glomerata	Biotroph	Powdery mildews	Sullivan and White, 2000
Eudarluca caricis	Biotroph?	Rust	Nischwitz et al., 2005
Cladosporium tenuissimum		*Uromyces appendiculatus*	Assante et al., 2004
Sphaerellopsis filum		*Melampsora* rusts	Bayon et al., 2006; Pei et al., 2003
Microsphaeropsis ochracea		Apple scab *Venturia inaequalis*	El Bassam et al., 2002
Clonostachys rosea	Epiphyte; bio- or necrotroph?	*Phytophthora palmivora*; *Moniliophthora roreri* on cocoa	Hoopen et al., 2003
Gliocephalis hyalina	Biotroph	*Fusarium*	Jacobs et al., 2005
Gliocladium catenulatum	Necrotroph?	*Aspergillus flavus*	Joshi et al., 1999
Paecilomyces fumosoroseus	Biotroph?	*Sphaerotheca fuliginea* powdery mildew	Kavkova and Curn, 2005
Parasitella parasitica	Biotroph? Gene transfer	*Absidia glauca*; Zycomycetes	Kellner et al., 1993; Schultze et al., 2005
Periconia byssoides	Necrotroph?	*Fulvia*, *Fusarium*, *Rhizoctonia*	Lin et al., 2007
Piptocephalis virginiana	Biotroph	Mucorales, specific hosts	Manocha, 1981; Manocha et al., 1997
Fusarium chlamydosporum	Necrotroph?	Rust *Puccinia arachidis*	Mathivanan et al., 1998
V. biguttatum	Biotroph	*Rhizoctonia solani*	McQuilken and Gemmell, 2004; Van Den Boogert and Deacon, 1994
Corniothyrium minitans	Necrotroph	*Sclerotinia*, *Sclerotium*	McQuilken et al., 2003; Rogers et al., 2004; Viterbo et al., 2007a
Stachybotrys elegans	Necrotroph?	*Rhizoctonia*	Morissette et al., 2006
C. spirale	Necrotroph	*Rhizoctonia*	Gao et al., 2005
Pythium periplocum	Necrotroph	*Botrytis cinerea*	Paul, 1999
P. oligandrum	Necrotroph	Other *Pythium*; sclerotial fungi	Picard et al., 2000; Rey et al., 2005
Pythium nunn	Necrotroph	Other *Pythium*	Elad et al., 1985; Viterbo et al., 2007a
Schizophyllum commune	Necrotroph	Wide	Tzean and Estey, 1978
Talaromyces flavus	Necrotroph	*Rhizoctonia solani*, *Sclerotium*, *Verticillium*	Fahima and Henis, 1990; Viterbo et al., 2007a
Trichoderma (Gliocladium) virens	Necrotroph	Wide	Harman et al., 2004; Papavizas, 1985; Mukherjee et al., 1995; Howell et al., 1993; Harman and Kubicek, 1998
T. atroviride, *T. harzianum*, *T. hamatum*	Necrotrophs	Wide	Chet and Inbar, 1994; Herrera-Estrella and Chet, 1998; Benitez et al., 2004; Harman and Kubicek, 1998

extracellular matrix originating from *R. solani* hyphae that was rich in galactose residues. This finding suggested the presence of receptors with galactose binding affinity at the cell surface of *Trichoderma*. Similarly, a matrix rich in chitin residues, originating from the pathogen, was detected in the interaction between *Pythium oligandrum* and *Fusarium oxysporum* f. sp. *radicis lycopersici* (Benhamou et al., 1999). Another necrotrophic mycoparasite, *Chaetomium spirale*, has been the object of a detailed study during its interaction with *R. solani* (Gao et al., 2005). The early recognition events are mediated by a β-1,3-glucan-enriched amorphous matrix (in this case originating from the mycoparasite) that triggers the firm binding of the antagonist to the host cell surface followed by pathogenic cell alterations including wall appositions, cell wall degradation, and plasmalemma and cytoplasm disorganization. The wall appositions are mainly composed of β-1,3-glucans that might function as defense barriers, similar to callose appositions in plants.

Trichoderma spp. not only parasitize the hyphae of many fungal species but also can penetrate and destroy some of the resting structures, thereby reducing their inoculum potential in soil. Soil pathogens produce resting structures known as sclerotia, which have a strong resistance both to chemical and biological degradation and permit the fungi to survive in the absence of a host. Sclerotia are composed of an outer rind layer of melanized cells that are thought to be responsible for resistance to microbial degradation in soil. *T. virens* aggressively parasitizes sclerotia as was shown first by SEM (Mukherjee et al., 1995) and later by fluorescence microscopy of green fluorescent protein (GFP)-expressing lines (Sarrocco et al., 2006) (Fig. 1C). Fluorescence microscopy revealed intracellular growth of the antagonist in the cortex

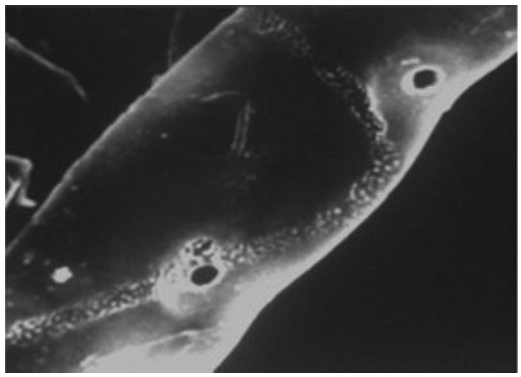


FIGURE 2 Image of holes in host cell walls left by *Trichoderma*, evidence of digestion by secreted enzymes. (Reprinted with permission from Elad et al., 1983b.)

(*Sclerotium rolfsii*) and intercellular growth in the medulla (*S. rolfsii* and *Sclerotinia sclerotiorum*). The uniformly distributed mycelium of *T. virens* just beneath the rind of sclerotia of both *S. rolfsii* and *S. sclerotiorum* suggests that the sclerotia became infected at numerous randomly distributed locations, without any preferential point of entry.

In a biotrophic mycoparasitic relationship, the host supports the growth of the parasite for an extended period without appearing to be diseased. The growth of the host and its ability to sporulate are not greatly affected in the early stages of infection. *Verticillium biguttatum* is an example of a biotrophic mycoparasite. From germinating spores, it penetrates the hyphae of *R. solani* and forms haustorium-like branches without killing the host cells (Fig. 1D). The haustoria support an external mycelial network of the mycoparasite. Later the mycoparasite sporulates, and the infected host cells die (Van Den Boogert and Deacon, 1994).

MYCOPARASITIC DEVELOPMENT: SECRETION OF ENZYMES

Fungus-fungus interactions are a unique class, distinguished from fungus-plant interactions, which are probably the best studied, or fungus-animal interactions, which are most important from a medical point of view. Many fungal species are saprophytes and are not pathogens of a specific host or class of host. What makes a saprophyte different from a pathogen, or a plant pathogen different from a fungus that causes disease in humans? The study of fungal genomes is beginning to help answer these questions. Interestingly, even saprophyte genomes encode enzymes that would be needed to breach the outer layers (cuticle, cell walls, and skin) of hosts. Indeed, in a comparative study where secreted enzyme activities were studied across lifestyles from saprophytes to plant, mammalian, and insect pathogens (St Leger et al., 1997), it was concluded that saprophytes and opportunists produced the broadest spectrum of both protein- and polysaccharide-degrading enzymes. The genome of the opportunist *Aspergillus fumigatus* encodes the enzymes needed to degrade plant substrates. Study of the proteome shows that efflux pumps and antioxidant enzymes are expressed, allowing this fungus to grow in a human host (Tekaia and Latge, 2005). Pathogens may have diversified from an ancestral saprophyte or opportunist (St Leger et al., 1997). Pathogens show enzymatic adaptation to the polymers that make up the cuticles of their hosts. St Leger et al. (1997) assayed the degradation of chitin by secreted enzymes of insect and plant pathogens. While insect pathogens need chitinase to attack their hosts, no detectable chitinase activity was secreted by the plant pathogens. These, however, were represented in the 1997 study by only a few *Verticillium* and *Nectria* isolates, a small sample of the vast diversity of plant pathogenic fungi. The CAZy database (Cantarel et al., 2009) shows 14 entries for GH18 family glycosyl hydrolases in rice blast, and there is evidence that some are indeed secreted (D. Ebbole, personal communication). The *Ustilago maydis* secretome, likewise, includes at least one chitinase. Its function in the plant-pathogen interaction is not clear; secreted chitinase is proposed to be involved in modifying the fungal cell wall (Mueller et al., 2008).

Plant pathogens are well adapted to the cell wall structure of their hosts. For example, the pattern of enzyme production by pathogens whose hosts are dicots or monocots reflects the different cell wall structures of these plants (Cooper et al., 1988). F. oxysporum, best known as a plant pathogen, can also infect mammalian hosts. Mutants lacking either the pathogenicity-related MAPK or the heterotrimeric G-protein subunit Fgb1 in this fungus lose virulence on plant hosts. The MAPK pathway was less important in infection of immunodepressed mice, while loss of Fgb1, which is required for secreted protease production had more of an effect, and virulence was lost in the double mutant (Prados-Rosales et al., 2006). Proteases are thus important for infection of animal hosts, while a repertoire of cell wall-degrading enzymes is needed to attack plants.

Assuming that these general principles apply to mycoparasites, one would expect to find secretion of chitinolytic enzymes, since chitin is a major component of host fungal cell walls (though not of Oomycetes, which are also hosts to *Trichoderma* and other mycoparasites [Table 1]). Accordingly, chitin-degrading enzymes have long been a focus of attention. An extensive analysis was done for *T. virens* (Kim et al., 2002). Upon completion of genome sequencing of *T. reesei*, Seidl et al. (Seidl et al., 2005) did a complete survey of chitinases in *T. reesei* and cloned five chitinases from *T. atroviride* biocontrol strains. Genomic analysis of hydrolytic enzyme encoding genes is presented in detail in chapter 27. An attractive hypothesis is that enzymes unique to mycoparasites might be important for the attack of fungal hosts (de la Cruz et al., 1995; Klemsdal et al., 2006; Lora et al., 1995; Seidl et al., 2005). Unique enzymes could be produced because the genes encoding them are found specifically in the genomes of mycoparasitic species or strains. Alternatively, specific genes might be expressed under mycoparasitic conditions.

Genomic analysis has already provided some support for the first alternative: *CHI18-15* of *H. jecorina* (which does not actually appear to be a mycoparasite in nature) has homologs in other *Trichoderma* species and in the insect pathogen *Cordyceps bassiana*, but not in other fungal genomes that have been studied. Insect hosts, like fungi, have a chitin-based barrier that must be overcome by a pathogen before it can obtain nutrients. All of the chitinases that contain cellulose-binding domains fall in group B, which is split into two subclades, B-I and B-II. *CHI18-13*, whose expression increases strongly under mycoparasitic conditions, belongs to clade B-I, with a closely related *Metarhyzium anisopliae* homolog, while the closest *T. virens* homologs are more distant, in clade B-II. Another mycoparasitism-dependent gene, *CHI18-10*, belongs to group C, and there is no *T. virens* homolog in this group at all, though further analysis of the complete genomic sequence

could identify more chitinase genes. Thus, correlations are emerging between mycoparasitic ability and specific chitinase genes, and also with the expression of these genes, combining both alternative hypotheses mentioned at the start of this section (Seidl et al., 2005). Further support will need to be obtained by constructing knockout and overexpressing mutants. The idea that the potential of mycoparasites (and pathogens of insects) to degrade host chitin is already encoded in the genome, rather than programmed by the expression of genes that are common to fungi that inhabit all niches, can now be explored further. Chitinase genes may have been duplicated and diversified beginning with those present in an ancestral saprophyte genome. The correlation between enzyme production and the composition of the cell walls of the host is not, however, always straightforward: antagonism of *Pythium* by *T. reesei* (*H. jecorina*), for example, does not depend on production of cellulase, even though the walls of this host are cellulose based (Seidl et al., 2006). It should be kept in mind, however, that *T. reesei* has not been found in soils that suppress plant pathogens. The correlation between enzymes and host cell walls might, then, prove to be more robust in host-mycoparasite pairs that have coevolved in the rhizosphere in nature.

Degradation of host cell walls is complex, requiring the concerted action of β-glucanases, cellulases, chitinases, and proteases (see Steyaert et al., 2003). Lytic enzymes are discussed in general and in the context of mycoparasitism, in chapter 27. In the framework of the *Trichoderma* expressed sequence tag (EST) project, endopeptidase genes, several of them novel, were identified, including members of the serine, aspartic, metalloendopeptidase, and aminopeptidase families. The observation that these genes are differentially regulated depending on culture conditions suggests that the encoded enzymes have specific functional roles (Suarez et al., 2007). When grown on insect cuticle, a broad-host-range isolate of the insect pathogen *M. anisopliae* expressed an unusually diverse set of secreted subtilisins (Bagga et al., 2004). This precedent suggests that the same type of selective pressures may have acted during the evolution of mycoparasitic fungi and that further work along these lines will uncover mycoparasitism-specific proteases.

GENE EXPRESSION IN MYCOPARASITISM: ENZYMES, "MARKER" GENES, AND CELL SURFACE PROTEINS

As discussed in the preceding section, secreted enzymes play a central role in the ability of the mycoparasite to obtain nutrition by degrading the fungal host. However, mycoparasitic development is more complex, involving the formation of new structures (see "Mycoparasitic Development: Coiling and Other Infection-Related Structures" above). From the gene expression point of view, there may be master regulators programming mycoparasitic development and expression of genes for hydrolytic enzymes. Alternatively, these two pathways might be only partly coupled. In order to distinguish between these possibilities and to better understand mycoparasitic development as a whole, several studies have followed an unbiased approach to identify the set of genes that are expressed during mycoparasitism. An obvious initial difficulty is that the parasite (pathogen) and host are both fungi. Perhaps motivated by the concern for isolating only mycoparasite genes, the first experiments compared *Trichoderma* grown as a saprophyte on glucose with growth on nonliving material of the host (e.g., autoclaved cell wall preparations). This experimental design (Chet and Baker, 1981) is referred to as "simulated" mycoparasitism. Such experiments led to the isolation of a basic protease gene (*PRB1*), which is expressed on autoclaved host mycelia, cell walls, or chitin, but not on glucose (Geremia et al., 1993). The endochitinase *ECH42* is induced under mycoparasitic conditions (Carsolio et al., 1994), as is the *N*-acetyl-β-D-glucosaminidase gene *NAG1* (Mach et al., 1999). Wider approaches based on differential screening led to the identification of genes expressed during growth on *Rhizoctonia* cell walls (Vasseur et al., 1995). In a more recent study, subtractive hybridization identified genes whose expression correlated with the mycoparasitic interaction of *T. hamatum* with *S. sclerotiorum* in a dual-culture plate confrontation, rather than simulated mycoparasitism on nonliving substrates. Nineteen novel *T. hamatum* genes were upregulated during mycoparasitism. The identified genes include the Woronin-body protein *HEX1*, monooxygenases, a metalloendopeptidase, a gluconate dehydrogenase, an endonuclease, and a proton ATPase (Carpenter et al., 2005). By a similar approach, genes whose expression is upregulated during interaction between the mycoparasite *Stachybotrys* and its host *R. solani* were identified. A large number of clones were obtained from the library, and sequencing of a few hundred produced 94 unique genes, of which about one-half were novel. Among those whose function could be predicted were genes related to toxin production, pathogenicity, and metabolism. Some upregulated host genes were also detected (Morissette et al., 2008). Partly because of the way the earlier studies were designed, there is overlap between host-induced genes and those induced upon release of catabolite repression. This overlap may be biologically meaningful; the mycoparasite, growing initially as a saprophyte in a nutrient-scarce environment, may express genes that will enable it to attack a host whenever it becomes available. A carbon catabolite derepressed *cre1* mutant of *H. jecorina* (*T. reesei*) was a better antagonist of *Pythium ultimum* than the wild type (Seidl et al., 2006). EST projects (Liu and Yang, 2005), including a major consortium (TrichoEST) (Vizcaino et al., 2006, 2007), are providing a very large number of genes expressed under various mycoparasitism-related conditions. These genome-wide studies do not seem, as yet, to have pinpointed any master regulatory genes but, rather, large classes of target genes. As bioinformatic tools become more powerful, shared regulatory elements upstream of these target genes may lead to transcription factors specific to mycoparasitic development or enzyme secretion. The smaller experiments employing differential library screening (by suppression subtraction hybridization [SSH] or other methods) remain relevant, as they can guide the analysis of the larger data sets. The challenge will be to choose which individual genes are the best high-priority candidates for knockout experiments. Such studies will lead to fundamental understanding of mycoparasitic development, as well as (hopefully) to improved biocontrol strains. Genes induced during the interaction may be excellent markers. It is important to remember, though, that manipulation of single genes sometimes (Flores et al., 1997), but not always (Carsolio et al., 1999), leads to a better strain.

Another approach will be to search in silico for upstream regulatory elements that are common to classes of coregulated genes and from there identify the master regulators. This is not straightforward yet with existing tools and data, but progress in identifying regulatory elements has been made for single genes, providing a reference point for future genome-wide analysis. Several

regulatory elements were predicted from the sequence of the promoters of the basic protease (*PRB1*) and endochitinase (*ECH42*) genes mentioned above (Cortes et al., 1998). The promoters of these two genes from *T. atroviride* share binding sites for the carbon response regulator CreA (four in *ECH42* and one in *PRB1*). *ECH42* has seven GATA sites and *PRB1* has two; these could mediate nitrogen regulation. A CCCCT stress response element is found in both genes. In addition, several elements are shared between the two genes, denoted MYC1 through MYC4: GCTTCA, TTGGCAA, GGGCAC, and GGCAWTCGGCAT, respectively (Cortes et al., 1998). These "mycoparasitism" elements were renamed MYRE1 through MYRE4, and additional sequences were identified (Olmedo-Monfil et al., 2002). The MYRE elements are also found in the *T. hamatum* orthologs (Steyaert et al., 2004). Binding sites for the pH regulatory transcription factor PacC are also found in these genes, and the ability to respond to ambient pH is important for mycoparasitism (Moreno-Mateos et al., 2007).

SIGNAL TRANSDUCTION AND MODULATION OF GENE EXPRESSION BY SIGNALS DERIVED FROM THE HOST FUNGUS

Signal Transduction Pathways

The proteins that make up the regulatory cascades are encoded by highly conserved genes. Signal transducers may be expressed more under developmental conditions where they are active, but this is not necessarily so; they can also be present constitutively and relay specific signals at critical times. Based on the high conservation of these genes among eukaryotes, it was quite straightforward to identify G-protein subunit and MAPK genes in a number of fungi, including species of *Trichoderma*. Pharmacological evidence that G proteins might be involved came from the finding that cyclic AMP (cAMP) as well as aluminum fluoride and mastoparan could increase coiling (Omero et al., 1999). In the Gα subunit nucleotide binding site, AlF_4^- mimics the γ phosphate of GTP, forming an active GDP-AlF_4^-/Mg^{2+} complex, so that the Gα subunit is permanently activated in the presence of GDP. Mastoparan, a wasp venom peptide toxin that activates heterotrimeric G proteins, bypasses the receptor(s). Mastoparan increased intracellular cAMP levels in a concentration-dependent manner and activated the GTPase activity associated with membrane fractions from *T. atroviride* (Rocha-Ramirez et al., 2002). Thus, the simplest interpretation was that host signals activate G-protein-coupled receptors (GPCRs), resulting in activation of a stimulatory G protein, in turn activating the mycoparasitic program. This could be true for coiling; however, caffeine, AlF_4^-, and dinitrophenol, all predicted to increase cAMP, repress rather than induce NAGase gene expression and protein accumulation in *T. harzianum* (Silva et al., 2004). Loss of the *GNA1* (originally named *TGA1)* gene of *T. atroviride*, encoding a G-protein α subunit, results in constitutive sporulation and elevated cAMP levels (Reithner et al., 2005; Rocha-Ramirez et al., 2002). Not surprisingly, these mutants, which grow very slowly and seem to devote most of their resources to making spores, have difficulty in overgrowing host fungi in confrontation assays. Interestingly, however, *gna1* (*tga1*) mutants of *T. harzianum* are actually better than the wild type at inhibiting the growth of host fungi, by overproduction of soluble metabolites. The inhibitory metabolite(s) remain to be identified (Reithner et al., 2005). However, it cannot be 6-pentyl-α-pyrone or sesquiterpenes, as these were underproduced in the mutant, indicating that this G-protein pathway affects biosynthesis of different metabolites in opposite ways.

Loss of another G-protein α subunit gene, *GNA3* (ortholog of *Neurospora gna-3*), like *GNA1* (*TGA1*) (ortholog of *Neurospora gna-1*) also leads to hypersporulation. The *gna3* (*tga3*) mutants were unable to overgrow several plant pathogens in confrontation assays and lost the ability to attach to host hyphae in dual culture but had enhanced levels of two chitinolytic enyzme transcripts (Zeilinger et al., 2005). Sporulation of *gna3* mutants was uncoupled from peptaibol production, which normally does accompany sporulation (Zelakowska-Komon et al., 2007). Thus, Gna1 and Gna3 (Tga1 and Tga3) apparently have overlapping roles in mycoparasitic development. How two or more heterotrimeric G-protein pathways might modulate cAMP levels is not yet clear. However, since it has been demonstrated that Gna1 influences adenylate cyclase activity, while Gna3 affects levels of adenylate cyclase protein in *N. crassa* (Kays et al., 2000), the same may be true in *Trichoderma*.

Overexpression of a cAMP-dependent protein kinase (PKA) regulatory subunit, which should decrease sensitivity to cAMP, causes conidiation in the dark (Casas-Flores et al., 2006). However, in *gna1* (*tga1*) mutants, with elevated cAMP levels conidiation is increased, seemingly the opposite effect expected from the correlation between cAMP and sporulation. Drastic reduction (or indeed elimination) of intracellular cAMP by knockout of adenylate cyclase leads to slow growth and loss of mycoparasitism in *T. virens*. However, induction of conidiation by light could still be observed in the mutants (Mukherjee et al., 2007). So is cAMP a positive or negative signal? Earlier biochemical work on *Trichoderma* suggests that there is a concentration window (Sestak and Farkas, 1993). Concentration and timing of cAMP localization in the cell may be key to understanding the mechanism. Genetically encoded indicators (cameleons) of cAMP concentration have been used in animal cells, but there have been difficulties in observing cameleon fluorescence resonance energy transfer (FRET) signals in fungi.

MAPK pathways have been studied by the same general approach, i.e., construction of loss of function mutants. Mutation of MAPK genes homologous to the pathogenicity-related MAPK of *Magnaporthe grisea* Pmk1 (Xu and Hamer, 1996) affects development and virulence of plant pathogenic fungi. The wide-reaching effects of loss of this gene in plant pathogens encouraged construction of the cognate mutant in *Trichoderma* species. Mutants of *T. virens* (Mendoza-Mendoza et al., 2003; Mukherjee et al., 2003) and *T. atroviride* (Reithner et al., 2007) sporulate in both light and dark. This is in contrast to other fungi, where sporulation is often decreased. Loss of *TMKA/TVK1* did not affect overgrowth of *R. solani* in confrontation assays but decreased antagonism against, and parasitism of sclerotia, of *S. rolfsii* (Mukherjee et al., 2003). In biocontrol against *R. solani* on cotton there was a significant decrease (30% of wild-type level) in disease index, and against *P. ultimum* the mutants were much more effective than wild-type *T. virens* (Mendoza-Mendoza et al., 2003). This could be explained by increased expression and induction of mycoparasitism-related genes in the mutant, in a host-dependent manner. In *T. atroviride*, mutants in the ortholog of the same MAPK gene, *tmk1*, showed increased coiling but reduced mycoparasitism in confrontation assays. Nevertheless, the mutant protected bean plants better than the wild type did, perhaps as a result

of increased production of chitinases or peptaibols (Reithner et al., 2007). The seemingly conflicting phenotypes of mutants in *PMK1* orthologs will need to be reconciled, as a function of (i) how biocontrol or mycoparasitic potential is assayed, (ii) host specificity, or (iii) *Trichoderma* species and strain variations.

So far, one other MAPK gene (of the three or four identified in ascomycete genomes) has been disrupted, the stress-activated protein kinase gene (*HOG1* ortholog) in *T. harzianum* (Delgado-Jarana et al., 2006). Loss of this gene led to stress sensitivity, as expected from the roles of Hog1p in yeast and other fungi, primarily to hyperosmotic stress, with a less pronounced increase in sensitivity to hydrogen peroxide. In cross-protection experiments, loss of *hog1* did not affect the ability of either osmotic or oxidative stress pretreatments to increase resistance to oxidative stress. Expression of an activated allele, however, led to the same high resistance without pretreatments (Delgado-Jarana et al., 2006). Antagonistic activity (measured in confrontation plate assays) against two of five tested plant pathogens was reduced, suggesting that osmolytes or reactive oxygen species (ROS) may accumulate during the mycoparasitic interaction. Hog1 homologs are needed for stress resistance, virulence, and differentiation of other filamentous fungi (Igbaria et al., 2008; Kawasaki et al., 2002; Kojima et al., 2004; Segmueller et al., 2007; Zhang et al., 2002). In fungus-plant interactions, accumulation of ROS is one of the plant's defenses, but this may not inhibit the fungal pathogen and actually favors attack by necrotrophs (Govrin and Levine, 2000). Similar experiments could be designed to compare necrotrophic and biotrophic mycoparasites. A *T. atroviride* strain engineered to secrete glucose oxidase was a better biocontrol agent than the wild type (Brunner et al., 2005). This suggests that production of ROS may be needed to attack the host. On the other hand, ThHOG1 may be needed to control ROS damage during a developmental transition in which ROS accumulates (Aguirre et al., 2005; Takemoto et al., 2007). In the plant pathogens, superoxide-generating NADPH oxidases are virulence factors (Egan et al., 2007; Giesbert et al., 2008; Segmueller et al., 2008). It seems likely that the study of fungus-fungus interactions will benefit here from what is known from phytopathology.

In the design and interpretation of experiments on signal transduction, it is important to note that signaling may be required for important cellular functions: the ability to grow at optimal rate, acquire nutrients, branch, or sporulate. Thus, loss of signaling may have secondary effects on mycoparasitism; so, to understand and manipulate signaling pathways that are relevant to fungus-fungus interactions, it will be necessary to identify an actual signal derived from a host fungus. This is not easy because receptor genes are not so well conserved, but it will become feasible with the increasing number of sequenced fungal genomes, as discussed in the next section.

Signals, Ligands, and Receptors

Identification of signals and receptors has proved more elusive than finding well-conserved signaling proteins. In the former case, there is no immediate way to guess what the signal might be, since the host can provide a multitude of signals ranging from surface properties, altered parameters like local pH, secondary metabolites, specific surface molecules, and small molecules released from the host by the action of the mycoparasite before or after contact. With respect to receptors, GPCRs are not very well conserved; now that genomic sequences are available, it has become feasible to identify them bioinformatically, and the first mutants are being studied in some saprophytes and fungal pathogens of plants and animals (Galagan et al., 2003; Kulkarni et al., 2005; Lafon et al., 2006; Xue et al., 2006). In *T. atroviride* four genes encoding GPCRs predicted to belong to the cAMP-sensing receptor class were cloned. These genes are all expressed at different levels, depending on growth conditions. For two of these cAMP sensors, silenced mutants were generated. Mutants in *gpr1* could not overgrow host fungi, while *gpr2* mutants show no phenotypic alterations compared to the wild type when grown alone and upon confrontation with living host fungi (Brunner et al., 2008).

The ligands for GPCRs (or other cell surface or intracellular receptors) have not yet been identified, though there are some indications of possible directions to pursue. Lectin-carbohydrate interactions (see "Mycoparasitic Development: Coiling and Other Infection-Related Structures" above) may provide one, but probably not the only, host signal. In an approach to find whether signals could already act at a distance from the host, Cortes et al. (1998) found that proteinase *PRB1* and endochitinase *ECH42* were induced in dual cultures, even when direct contact with the host was prevented by a cellophane membrane separating the mycoparasite and host colonies. Neither gene was induced during the interaction of *Trichoderma* with lectin-coated nylon fibers, so that the induction of coiling and of the expression of these genes apparently depends on distinct signaling pathways. A diffusible, heat-stable molecule derived from *R. solani* cell walls induced expression of both genes.

Using GFP as a reporter, Zeilinger et al. (1999) and Viterbo et al. (2002) showed induction of some chitinase genes by host-derived diffusible signals (Fig. 3). Other genes, for example, *NAG1*, are expressed only after physical contact (Zeilinger et al., 1999). Furthermore, *NAG1* expression is triggered by the chitin mono- and oligomers *N*-acetylglucosamine, di-*N*-acetylchitobiose, or tri-*N*-acetylchitotriose, while endochitinase *ECH42* is not (Mach et al., 1999). Digestion of *R. solani* cell walls by *Trichoderma* or by enzymes released inducer fractions (Zeilinger et al., 1999). The identification of these may provide an entry point to elucidating the host signals and their receptors.

An attractive model (Fig. 4) is that some enzymes are expressed at low levels, perhaps derepressed under nutrient limitation. A regulatory loop, in which the products of initial degradation of a polymeric substrate induce the expression of genes for larger-scale degradation, seems to be a general adaptation of filamentous fungi. An example is the induction of cellulases in *T. reesei* (el-Gogary et al., 1989). It was found that a gene encoding a secreted β-1,6 glucanase is expressed during growth on autoclaved mycelia of different host fungi (Lora et al., 1995). Because this gene is glucose repressed, the authors postulated that the enzyme may release elicitors when the mycoparasite grows under nutrient limitation and proposed constructing transgenic plants and biocontrol *Trichoderma* strains expressing this gene. Low-level hydrolytic enzymes expressed before contact with the host could digest, to some extent, the host cell walls, releasing inducers, which in turn switch on the more massive (and, presumably, energy-expensive for the cell) production of secreted enzymes (Fig. 4). The factor found by one study (Cortes et al., 1998) was heat stable and could traverse cellophane; Kullnig et al. (2000) found activity that could not pass dialysis

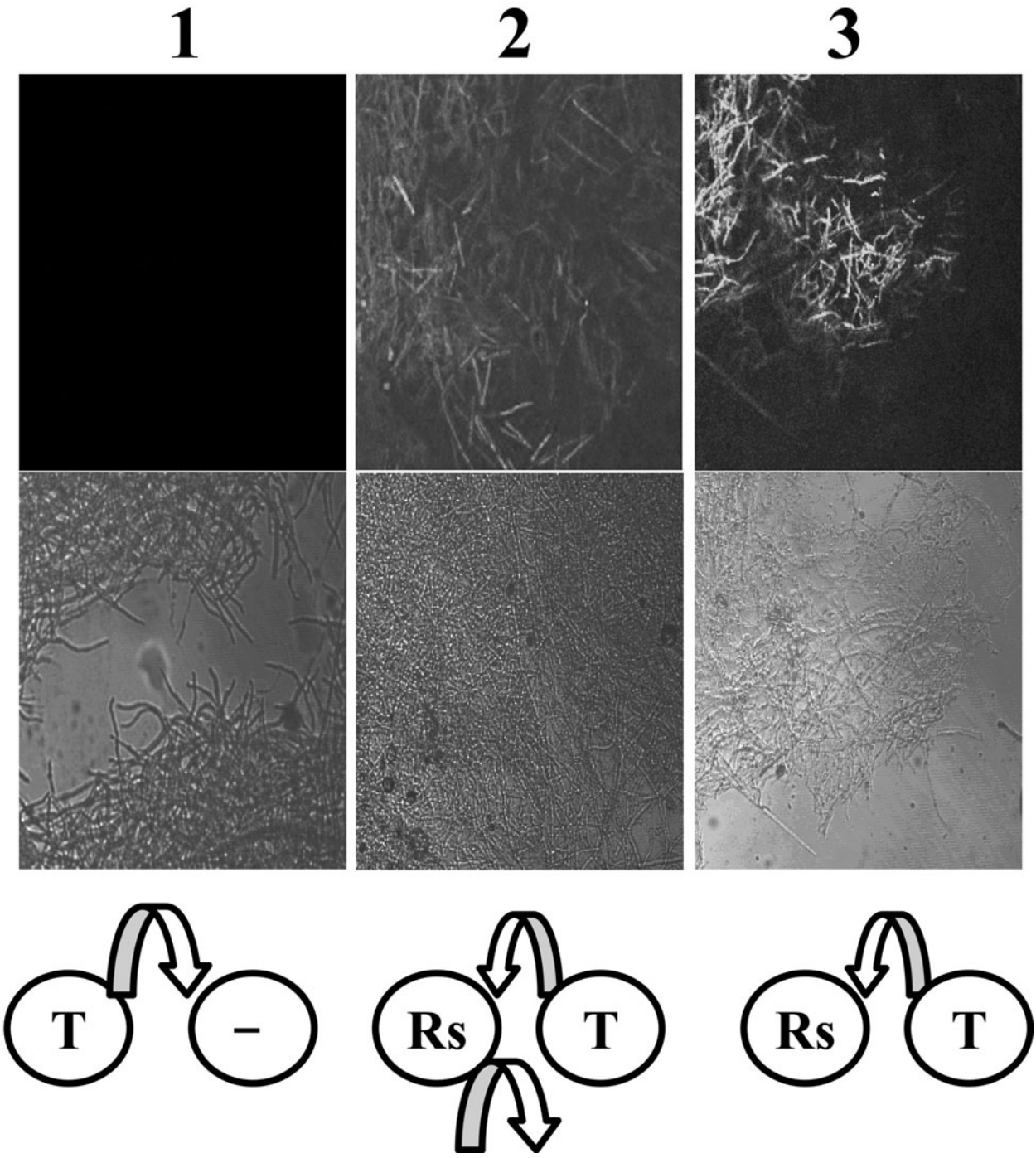


FIGURE 3 Modulation of mycoparasite gene expression by host-derived signals. Expression of chit36:GFP in a transgenic *Trichoderma* line (T) induced by interaction with *R. solani* (Rs). *Trichoderma* was grown on a dialysis membrane on minimal medium for 2 days and then transferred for 24 h onto a clean plate (panel 1); a plate where *R. solani* was previously grown for 2 days (panel 2); or a 2-day-old culture of *R. solani* (panel 3). (Reprinted from Viterbo et al., 2002, with permission of Springer Science and Business Media.)

membranes with appropriate cutoff and might be an enzyme. Another study (Brunner et al., 2003) showed that *NAG1* is essential for the induction of *ECH42*, placing *NAG1* upstream, yet the earlier experiments had shown that *NAG1* is induced only after contact. A low activity of Nag1 might be present constitutively in the earlier stages of interaction. The chemical identity of the postulated host-derived elicitors (Fig. 4) has been elusive. Initial evidence that the active molecules are chitin oligomers with an amino acid residue has been cited (Woo et al., 2006);

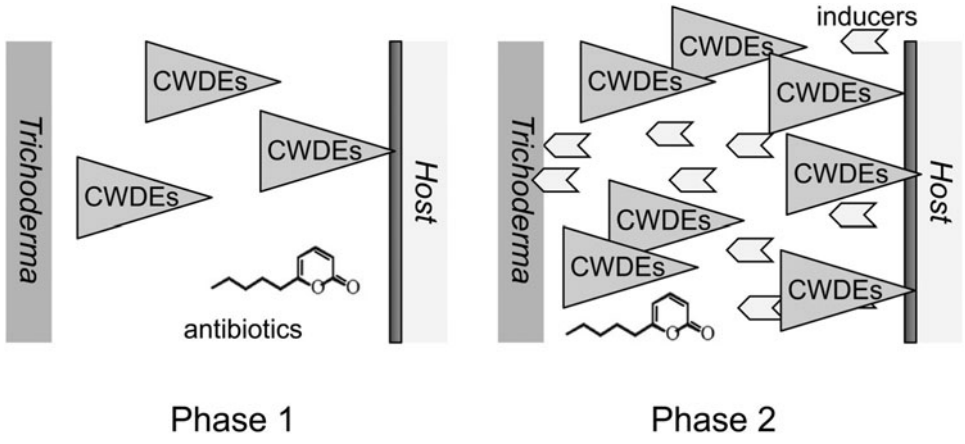


FIGURE 4 A model for how host signals are produced. Action of hydrolytic enzymes secreted by the mycoparasite releases diffusible products, and secondary metabolites of fungal origin (for example, 6-pentyl-2H-pyran-2-on3). These signals may, in turn, program the development of the mycoparasite. (Reprinted from Vinale et al., 2008, with permission from Elsevier.)

full structural elucidation and identification of the receptors that detect these molecules will be one of the most interesting directions to pursue.

THREE-WAY INTERACTION: PLANT AS A BYSTANDER DETECTING MYCOPARASITIC INTERACTION BETWEEN PATHOGEN AND BIOCONTROL FUNGI

The colonization of the rhizosphere by certain strains of plant growth-promoting rhizobacteria results in a state of heightened resistance to subsequent pathogen attack, a phenomenon generally known as induced systemic resistance (ISR) (van Loon, 2007). Although ISR is not actually part of the mycoparasitic interaction, it may be a consequence of it, for example, if inducers of ISR are released from the fungal host. In addition to plant growth-promoting rhizobacteria, anamorphic stages of several fungi, including *Trichoderma* spp., *Fusarium* spp., binucleate *Rhizoctonia*, and *Pythium oligandrum*, have been found to induce plant resistance to pathogens (Brozova, 2002). Mycoparasitic fungi that have developed the ability to interact simultaneously with plants and with fungal pathogens can be used as model microorganisms to study complex and multiple-player plant-microbe interactions (Fig. 5A). These fungi occur on/inside plant roots together with plant pathogens and do not attack plant tissues but utilize root exudates and fungus mycelia, including plant pathogens, for their own nutrition. These mycoparasites not only have direct effects, such as protection against attack by different plant pathogens in the rhizosphere, but also have a stimulating influence on plant growth and induction of defense responses.

Plants recognize microorganisms by a variety of mechanisms. Plant defense responses can be induced by oligosaccharide elicitors both of plant origin (pectin and cellulose or hemicellulose oligomers) and of fungal origin (chitin or glucan oligomers). The signals from plants are produced by cell wall-degrading hydrolases of fungi or bacteria, while those from fungi are generated by plant pathogenesis-related proteins (chitinases and glucanases) or fungal enzymes secreted during mycelial growth or mycoparasitic interactions with "bystander" plant root pathogens. By sensing the biocontrol agent metabolites, the plant may become aware of the "battle" eventually occurring in the rhizosphere and quickly activate the defense system before or immediately after the pathogen attack (Woo et al., 2006). Studies on the mechanisms of action used by *Trichoderma* spp. indicate that the stimulation of plant defense responses seems to be a major contributor to successful biocontrol (Harman et al., 2004). The plant interaction with *Trichoderma* normally increases the ability to withstand both biotic and abiotic stresses. De facto, the activity of these fungal biocontrol agents commonly determines the level of plant susceptibility or resistance to pathogens. The use of relatively novel tools to investigate these complex processes, such as proteomic analysis, use of gene expression reporter systems, and high-throughput methods to study gene function, has demonstrated that a molecular cross talk is established between *Trichoderma* spp., the plant, and the pathogen (Marra et al., 2006). Several studies have shown that root colonization by *Trichoderma* strains results in massive changes in plant gene and protein expression profiles (Alfano et al., 2007). A potentiation in the gene expression enables *Trichoderma*-treated plants to be more resistant to subsequent pathogen infection (Shoresh et al., 2005).

Just as the plant recognizes colonization of its roots, microorganisms in the rhizosphere recognize the plant. This may be particularly critical for the rhizosphere of germinating seeds, termed spermosphere (Nelson, 2004). Germinating seeds release compounds, including sugars and sugar alcohols, amino acids, aliphatic and aromatic organic acids, phenolics, lipids, flavonoids, volatiles, and others. The primary inoculum of plant pathogens is present in the soil, and the initiation of soilborne disease can depend critically on the signal provided by compounds released from the host plant. Oospores of *Pythium aphanidermatum* and chlamydospores and macroconidia of *Fusarium solani* (pea or bean pathogen species) have been shown to respond to seed exudates by germinating rapidly. *Pythium* detects long-chain fatty acids, while *Fusarium* may respond to flavonoids. An efficient way to apply biocontrol agents is by coating seeds. In the critical first 24 h of germination, rapid development of the pathogen is induced by plant exudate compounds. During the same time, an effective biocontrol agent must win the developmental race by responding to the same compounds that induce pathogen growth or different classes of exudate compounds released in the same time window

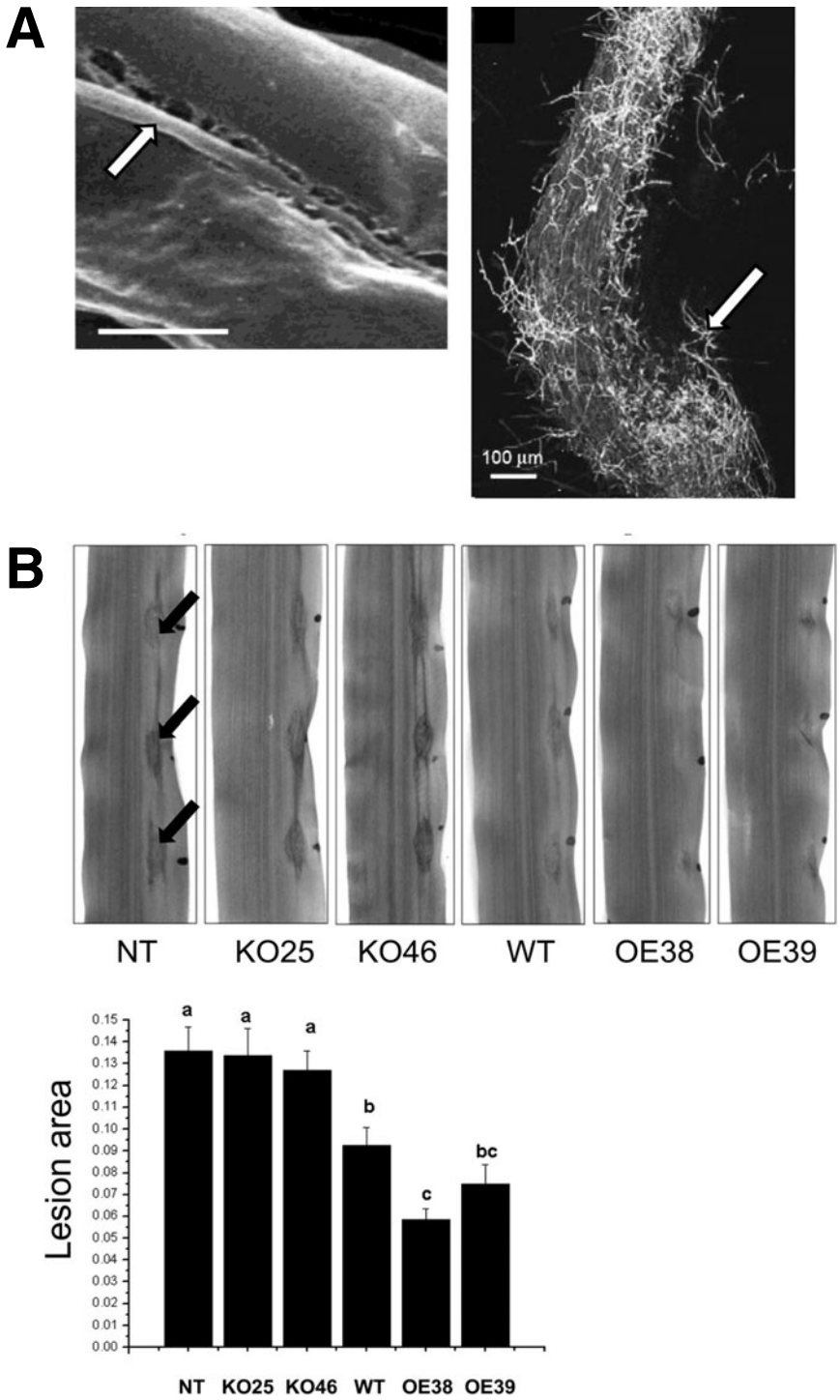


FIGURE 5 Interactions with plant roots. (A) Left, interaction between *T. asperellum* (hypha indicated in SEM image by the arrow) and cucumber root. (Reprinted with permission from Yedidia et al., 2000.) Right, interaction between tomato root and *T. harzianum* expressing GFP (fluorescent hyphae indicated by arrow). (Reprinted with permission from Chacon et al., 2007.) (B) Consequence of *Trichoderma*-root interactions: induction of systemic resistance in the plant. Bioassay for induced resistance in maize seedlings against *Colletotrichum graminicola*. Left, symptoms on leaves of maize seedlings grown from untreated (NT) seeds, or seed treated with knockout strains (KO25 and KO46), wild-type strain (WT), or overexpression strains (OE38 and OE39) of *T. virens*. Lesions (indicated, for example, by black arrows in left-most leaf photo) appear dark in this grayscale image; see Djonovic et al. (2007) for original color image from which this figure was reprinted with permission. The gene disrupted or overexpressed in these *T. virens* strains encodes the secreted elicitor protein Sm1. Right, quantitative analysis of lesion size for the experiment shown in the left panel; different letters indicate significant differences (Djonovic et al., 2007).

(Nelson, 2004). More attention, therefore, should be given to whether faster germination, specialized development (coiling), enzyme secretion, and antibiotic production by the mycoparasite might be induced by plant exudates, much as recent studies (see above) have addressed induction by chemical signals originating from the host fungus.

Signal transduction by *Trichoderma* spp. is required for full induction of ISR in the plant (Viterbo et al., 2005). The signals remain to be identified, and possible candidates would be root exudate compounds. It seems that fungal MAPK pathways may transduce plant signals, telling the fungus to release factors that are in turn detected by the plant. There are at least three classes of substances that could elicit ISR in the plant: proteins, peptides, and low-molecular-weight compounds (Harman et al., 2004; Woo et al., 2006; Viterbo et al., 2007b). Woo et al. (2006) define three classes of elicitors: (i) enzymes or peptides; (ii) homologs of the *AVR* genes of plant pathogenic fungi (the genes are named *AVR* for "avirulence," due to recognition of these proteins by the plant host in gene-for-gene interactions); and (iii) small inducer molecules released from the host fungus and plant cell wall by the cell wall-degrading enzymes of *Trichoderma*.

Changes in plant metabolism lead to the accumulation of antimicrobial compounds (Yedidia et al., 2003). Research has shown that there is a strong correlation between the ability of a strain of *T. virens* to induce terpenoid synthesis in cotton roots and its efficacy as a biocontrol agent of cotton seedling disease (Howell, 1998). Strains that do not induce terpenoid synthesis do not protect the root from subsequent infection by a pathogen. Apparently, induction of phytoalexin synthesis in the roots of the cotton plant also protects it from the vascular wilt pathogens that enter through the root. *T. virens* has been shown to produce a series of proteins and peptides that induce terpenoid phytoalexins involved in disease resistance in plants.

Two synthetic 18-amino-acid peptaibol isoforms (TvBI and TvBII) from *T. virens* Gv29-8 when applied to cucumber seedlings through the transpiration stream were shown to be able to induce systemic resistance and to induce antimicrobial compound synthesis in cucumber cotyledons (Viterbo et al., 2007b).

Purified protein Sm1, from the same strain, efficiently elicited plant defense responses and systemic resistance against a foliar pathogen of cotton (Djonovic et al., 2007). The protective activity of Sm1 was associated with the accumulation of transcription of the defense genes regulated by SA and JA/ET as well as genes involved in the biosynthesis of sesquiterpenoid phytoalexins. Sm1 has been shown by biochemical and reverse genetic analyses to be essential for resistance induced by this fungus. Sm1 is required for activation of ISR in maize, since plants grown with SM1 deletion strains exhibited the same levels of systemic protection as the control (non-*Trichoderma*-treated) plants and, conversely, plants grown with SM1-overexpression strains displayed increased protection compared to the wild type (Djonovic et al., 2007) (Fig. 5B).

CONCLUDING REMARKS

Fungus-fungus interactions provide a unique area of study. The mechanisms by which a fungal pathogen (mycoparasite) recognizes and colonizes its host may be similar in some ways to those used by pathogens of plants or animals. Indeed, because fungi are ancient, fungus-fungus interactions may have preceded the evolution of fungi as pathogens of plants and animals. Chytrids parasitizing vesicular arbuscular mycorrhizae were found in the fossil record of the Rhynie Chert, dating to more than 400 million years ago (Hass et al., 1994). There are theories that mycorrhizae themselves might have evolved from biotrophic fungal parasites of plants (Remy et al., 1994). These considerations lead one to speculate that the same cellular processes may underlie fungal disease and mycoparasitism. The latter is often beneficial (except to mushroom growers), but the application of what has been learned about plant and animal pathogens to the study of mycoparasitism should continue to yield insight. Major features such as detection of the host, signal transduction, altered transcriptional patterns, and secretion of enzymes are likely to be shared, and this has provided working hypotheses to guide studies of mycoparasitism.

The practical use of fungi as antagonists to soilborne plant pathogenic fungi has motivated most of the work on fungus-fungus interactions. The coming years will show to what extent basic research can improve biocontrol and what directions should be followed to best serve both basic interest and biotechnological relevance. A large portion of the molecular genetic work has been done on *Trichoderma*, while the full diversity of mechanisms used by biotrophic and necrotrophic mycoparasites remains to be explored. The precise timing of plant, pathogen, and mycoparasite development will be different for spermosphere (seed pretreatment), steady-state growth in field or greenhouse, or mycoparasitism of sclerotia in the soil. Molecular mechanisms and genes manipulated to optimize biocontrol will be different for each type of interaction and for each niche within the complex web of fungus-fungus and fungus-root interactions.

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ANIMAL PATHOGENS

XI

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Cellular and Molecular Biology of Filamentous Fungi
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Aspergillus fumigatus

JUDITH C. RHODES AND DAVID S. ASKEW

INTRODUCTION

Aspergillus fumigatus is a ubiquitous fungus that is found in high concentration in environments that are rich in decaying vegetation. In particular, *A. fumigatus* is an important primary consumer in the composting process, in which it plays a central role in the recycling of carbon and nitrogen (http://www.css.cornell.edu/compost/invertebrates.html). In this inhospitable environment, the fungus is subjected to various stressors, such as nutritional limitations, high temperatures, and acid pH. In adapting to survive in this ecological niche, *A. fumigatus* has acquired numerous metabolic and physiologic properties that assist in its adaptation to the mammalian host (Latge, 1999). For example, the thermotolerance of the fungus makes it ideally suited for rapid and robust growth, even during the high-temperature phase of the compost cycle, allowing *A. fumigatus* to outcompete many other organisms. This ability to grow effectively at elevated temperatures undoubtedly also contributes to its prevalence as an opportunistic pathogen (Araujo and Rodrigues, 2004). Likewise, survival in compost following the formation of humic acid and the subsequent generation of singlet oxygen may enhance the ability of *A. fumigatus* to survive the acidic environment and reactive oxygen species that it encounters in the host (Paul et al., 2004). In order to propagate in the environment, the fungus produces over 50,000 asexual spores, called conidia, per conidiophore (Mackenzie, 1988). These pigmented conidia are small and hydrophobic, and these properties make them easy to aerosolize and, subsequently, to be inhaled by humans and animals (Fig. 1). The outcome of this encounter with a mammalian host depends largely upon the host's immunologic status (Tekaia and Latge, 2005). However, if aspergillosis were the result of simple stochastic encounters between the host and the members of the genus *Aspergillus*, one would expect that the spectrum and prevalence of species obtained from air sampling would mirror that of isolates from the diagnostic laboratory. It is notable that the predominant clinical isolates are *A. fumigatus*, despite the fact that this species represents less than one-half of the airborne *Aspergillus* isolates (Hospenthal et al., 1998). These data suggest that *A. fumigatus* is endowed with specific properties that make it a more successful opportunistic pathogen, an observation that drives the research effort to understand the factors that contribute to the pathogenesis of aspergillosis. This need is accentuated by the progressive rise in prevalence in invasive disease seen in the past several decades (Bodey et al., 1992; Cagatay et al., 2008; Cornillet et al., 2006; Marr et al., 2002; Talbot et al., 2006; Upton et al., 2007; Wingard et al., 2008).

History

Both the organism *A. fumigatus* and the disease it causes have been known for approximately 150 years. Pier Antonio Micheli described the genus in 1729 in *Nova Plantarum Genera* (*New Genera of Plants*) and named it *Aspergillus* for its resemblance to a holy water sprinkler or aspergillum (Mackenzie, 1988). In 1863, Fresenius published the description of the species *fumigatus* in his monograph *"Beiträge zür Mykologie"* (*Contributions to Mycology*), which contained descriptions of over 100 new or revised fungal species (Schmidt and Schmidt, 1999). The first description of human aspergillosis, a term introduced by Fresenius, was provided by Virchow in 1856 in a classic article that contains excellent camera lucida drawings of organisms that are easily recognizable today as *A. fumigatus* (Mackenzie, 1988). Interestingly, Fresenius compares his isolate, from an avian infection, with Virchow's human isolate. Since that original description, infections of humans, other mammals, and many birds have been reported. Thus, in a relatively short period, *A. fumigatus* has become the most commonly reported opportunistic filamentous fungus (Marr et al., 2002; Talbot et al., 2006).

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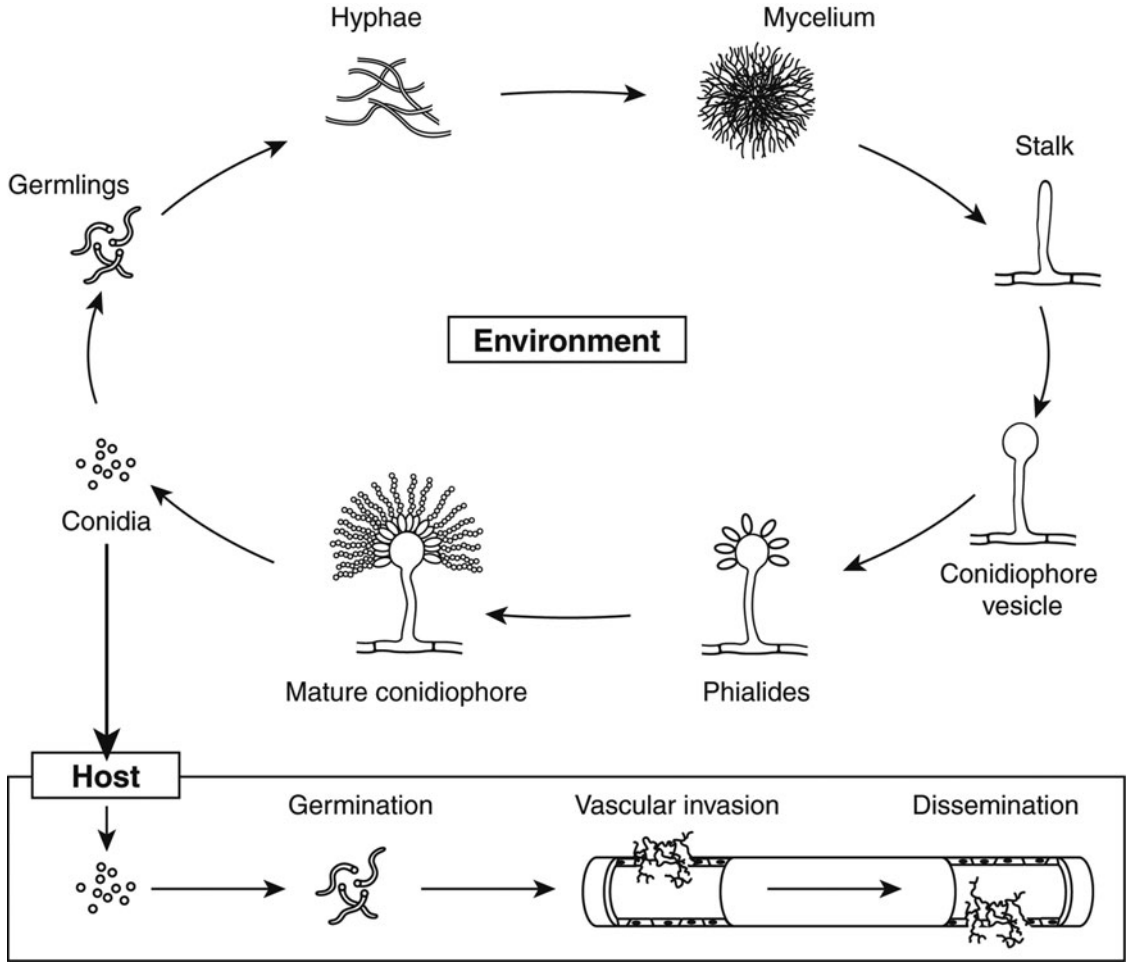

FIGURE 1 Schematic illustration of the life cycle and pathogenesis of *A. fumigatus* (not drawn to scale). *A. fumigatus* propagates itself in the environment by the release of asexual conidia into the atmosphere. The conidia begin the process of germination when they encounter a suitable substrate, forming mature hyphae that eventually develop into a mycelium. In response to an appropriate stimulus, such as nutrient deprivation, some of the hyphae activate the developmental program of asexual development, resulting in a series of morphological changes: an aerial hypha, or stalk, forms from a thick-walled foot-cell, and the stalk tip swells to form a vesicle. A single row of phialides then forms on the vesicle surface, from which the conidia subsequently develop. A mature conidiophore can possess up to 50,000 conidia, which are released into the environment in response to changes in airflow. The airborne conidia are inhaled by most people on a daily basis and are small enough (2 to 3 μm in diameter) to reach the distal airways. The conidia are efficiently cleared in a healthy individual, but in an immunodeficient host they are able to germinate into invasive hyphae that penetrate the vasculature and migrate to distal sites.

ASPERGILLOSIS

A. fumigatus causes a spectrum of clinical entities, ranging from allergic rhinitis to invasive disease. Although invasive infections with other *Aspergillus* species have been reported, *A. fumigatus* is responsible for approximately 90% of these cases. Casadevall and Pirofski have described a damage-response continuum to explain the complicated relationships between an organism and the host in which it causes disease (Casadevall and Pirofski, 1999, 2001, 2003). Disease is defined as the result of damage to the host, whether that damage is mediated by the organism or by the host in response to the organism. Using this damage hypothesis as a framework, disease is caused by *Aspergillus* at both ends of the continuum of host response.

Immune-Mediated Disease

Following the inhalation of conidia, allergic rhinitis and allergic bronchopulmonary aspergillosis (ABPA) result from overly exuberant immunological responses (Tillie-Leblond and Tonnel, 2005). In allergic rhinitis, an atopic reaction is triggered immediately by exposure to the conidia, causing symptoms referred to as "hay fever" or immediate-type hypersensitivity. Whereas atopy is the only recognized underlying factor in allergic rhinitis, ABPA is diagnosed most frequently in patients with asthma and patients with cystic fibrosis. In ABPA, the damage to the host is caused by the host's own immunological response to antigens on the fungus itself and in products elaborated by the fungus. In this case, the inhaled conidia are able to germinate in the excess

mucus in the respiratory tract, but the germlings do not penetrate the lung tissue. The persistence of the hyphae in the mucus plugs results in the accumulation of fungal products that are recognized by the host as foreign antigens. These antigens elicit an antibody-mediated, early immune response, followed by the development of granulomata as chronic inflammation continues unchecked (Soubani and Chandrasekar, 2002). Ultimately, the unresolved inflammation can lead to destruction of the lung parenchyma, with a decrease in the patients' abilities to perform the necessary gas exchange. Genetic studies suggest that susceptibility to ABPA among asthmatics may be determined by HLA, with HLA-DR2 and DR5 being associated with susceptibility and HLA-DQ2 being associated with resistance(Glimp and Bayer, 1981)). Diagnostic criteria for ABPA include clinical, radiographic, and laboratory findings. X-ray findings may be suggestive of ABPA, but the diagnosis is confirmed by the detection of antibody specific for *A. fumigatus*, especially when accompanied by high circulating immunoglobulin E levels (Kurup et al., 2006). The sensitivity and specificity of the serologic testing required for the diagnosis of ABPA have been facilitated in the past decade by the availability of specific recombinant *A. fumigatus* antigens; these reagents have allowed the serological identification of approximately 90% of positive patients. Treatment of ABPA patients is complicated by the fact that many of them are corticosteroid-dependent asthmatics. In early trials of polyene and azole antifungals, patients showed no improvement over steroids alone. Therapy trials conducted following the introduction of itraconazole, an oral antifungal with anti-*Aspergillus* activity, quickly showed that antifungal treatment was efficacious in ABPA, often resulting in a decreased requirement for steroids (Tillie-Leblond and Tonnel, 2005). In addition to its antifungal activity, itraconazole contributes to a dampening of immune activation, which leads to decreased eosinophilic inflammation and improved clinical outcomes (Wark et al., 2003). These findings have led to the inclusion of itraconazole as one of the key pharmacologic approaches to therapy of ABPA in asthmatics and in cystic fibrosis patients.

Aspergilloma

Aspergilloma or fungus ball is a form of aspergillosis that is often referred to as colonization, because it is initiated by *Aspergillus* growing in a preexisting cavity in the lung (Soubani and Chandrasekar, 2002). The cavity could be the result of cancer, tuberculosis, sarcoidosis, or another pulmonary disorder. A colony or fungus ball of *Aspergillus* may exist free in the cavity or become fixed to the cavity wall. In many cases, the patients are asymptomatic, but with time the cavity may expand and erode into blood vessels, which results in hemoptysis. At that point, surgery is often required, as drug penetration into the cavitary lesions is usually insufficient to effect a cure (Richardson and Kokki, 2003).

Invasive Disease

Whereas the allergic forms of aspergillosis are found at one end of the damage-response curve, invasive aspergillosis is found at the other (Casadevall and Pirofski, 1999). For the most part, invasive aspergillosis begins as a pulmonary infection of markedly immunocompromised hosts, such as patients suffering from hematological malignancies, receiving high-dose corticosteroid therapy, or following hematopoietic stem cell or solid organ transplantation (Burgos et al., 2008; Marr et al., 2002; Upton et al., 2007; Wingard et al., 2008). Among other risk factors for invasive aspergillosis are broad-spectrum antibiotic therapy, cytomegalovirus infection, and hereditary conditions such as chronic granulomatous disorder (Antachopoulos et al., 2007; Kontoyiannis and Bodey, 2002; Marr et al., 2002; Meersseman et al., 2007; Munoz et al., 2006). Interestingly, as has been shown for ABPA, host genetic factors may also play a role in susceptibility to invasive aspergillosis. In inbred mice and in a cohort of hematopoietic stem cell transplant recipients, nonsynonymous coding changes in the gene encoding plasminogen appear to correlate with relative susceptibility (Zaas et al., 2008). In humans, the minor allele (Asp472Asn) was found more frequently in the population with increased risk of acquiring invasive aspergillosis; the adjusted hazard ratio or 5.56 for homozygotes and 3.00 for heterozygotes suggests a gene dosage effect. Plasminogen was shown to attach to *A. fumigatus* swollen conidia and hyphae, possibly altering their further interactions with host cell and in inflammatory cascades (Zaas et al., 2008).

In an immunocompetent host, germination of inhaled conidia is inhibited by aggregates of neutrophils, and the conidia are phagocytosed and killed by alveolar macrophages (Bonnett et al., 2006; Ibrahim-Granet et al., 2003). Germlings that escape the initial host defense can also be killed by neutrophils (Schaffner et al., 1982). Susceptible hosts are deficient in these effectors of innate immunity and are, therefore, unable to kill the inhaled conidia or to prevent them from germinating. Once the hyphae penetrate the lung parenchyma, the microcolony expands in a spherical fashion, expanding without respect to anatomical borders. When it encounters a blood vessel, as shown in Fig. 1, it can penetrate the vessel wall, a process called angioinvasion, and gain access to the vasculature. From there, pieces of the fungus, probably encased in small clots or thrombi, will shear off and be spread throughout the circulatory system. Through this process of hematogenous dissemination, the fungus can lodge in any organ system and initiate new growth at remote sites. These disseminated infections carry a particularly high mortality, 70% or higher in some studies (Upton et al., 2007; Wingard et al., 2008). Compounding the clinical problem, infection in susceptible hosts is difficult to diagnose. The diagnosis involves a combination of clinical awareness, radiographic evaluations, particularly computed tomography, and culture and histology of infected tissue (Munoz et al., 2006). In the past decade, detection of circulating galactomannan, which is a sensitive and relatively specific marker of invasive aspergillosis, detection of β-glucan, and PCR methods have contributed to the more timely diagnosis of invasive aspergillosis in patients (Hope et al., 2007; Mennick-Kersten et al., 2008; Miceli et al., 2008). The galactomannan test has performed particularly well when used prospectively to monitor patients with hematological malignancies at risk of invasive aspergillosis (Hope et al., 2007; Miceli et al., 2008; Steinbach et al., 2007). Cross-reactivity to piperacillin-tazobactam and, potentially, other beta-lactam antibiotics that are synthesized by fungi closely related to *Aspergillus* has been the most significant problem in interpreting the test results (Aubry et al., 2006; Penack et al., 2008). Detection of *Aspergillus* DNA by use of amplification methods has been applied by many investigators to samples including blood, serum, and bronchoalveolar lavage fluid (Boudewijns et al., 2006). Although several combinations of specimens, processing methods, and primers sets have shown promise, amplification-based methods, as a whole, have been hampered by the lack of standardization, which has made comparison of results between centers difficult.

Although therapy of invasive aspergillosis has improved significantly in the past decade, the 1-year survival rate for

hematopoietic stem cell transplant recipients diagnosed with invasive aspergillosis remains less than 40% (Upton et al., 2007). There are three classes of drugs that are approved for the treatment of aspergillosis: polyenes, azoles, and echinocandins (Walsh et al., 2008). The polyenes, which include amphotericin B and its various lipid-associated forms, were the mainstay of therapy for decades, and they remain an important treatment modality for invasive disease. This class of drugs works by binding to ergosterol in the cell membrane and causing leakage of cell contents leading to death of the cell. Although the amphotericin B family of drugs remains active against *A. fumigatus*, its usefulness is limited by host toxicity, even in the lipid preparations. Voriconazole, an antifungal triazole that acts by inhibiting the 14-alpha-lanosterol demethylase required for ergosterol biosynthesis, has become the drug of choice for serious invasive aspergillosis (Walsh et al., 2008). Outcomes appear to be somewhat better with this triazole than with amphotericin B. Itraconazole continues to be used for prophylaxis against invasive disease in susceptible patients and for therapy in patients with ABPA. The structurally related azole posaconazole has been recently approved for prophylaxis of invasive disease. The third class of antifungals used for the therapy of invasive aspergillosis is the echinocandins, which inhibit β-glucan synthase, an essential enzyme for cell wall synthesis (Hu et al., 2007; Mouyna et al., 2004). Caspofungin is approved for therapy of invasive aspergillosis that is refractory to other therapy or when the patient is intolerant of other modalities. Although the in vitro data suggest that both micafungin and anidulafungin will be efficacious, there are as yet insufficient clinical data for these drugs. Although diagnostic tests and therapeutic options have improved, the morbidity, mortality, and pharmacoeconomic burden remain extraordinarily high, especially following dissemination of the organism from the lungs to other organ systems (Cagatay et al., 2008; Wilson et al., 2002; Wingard et al., 2008). These data, as well as the recent recognition of invasive aspergillosis in new patient populations, such as those in intensive care units and other critically ill patients without malignancy, continue to drive research efforts to identify key factors related to the pathogenicity of *A. fumigatus*, new diagnostic modalities, and potential targets for new therapeutic drug options (Talbot et al., 2006).

MOLECULAR TOOLS: GENOMICS AND MICROARRAYS

Transcriptional Profiling: Thermotolerance

Techniques for global analysis of transcription are revolutionizing our understanding of fungal physiology, including the complex regulatory networks that facilitate adaptation to the host environment. Although the application of this technology to the study of *A. fumigatus* biology and virulence is still in its infancy, a number of recent studies have used transcriptional profiling to ask important questions about gene function, as well as the response of the fungus to certain types of environmental stress. The first large-scale analysis of the *A. fumigatus* transcriptome was published in conjunction with the initial report of the Af293 genome sequence (Nierman et al., 2005). In this study, microarrays were used to gain insight into genes that are differentially regulated in response to a temperature shift, with the goal of understanding aspects of thermotolerant growth that are relevant to mammalian infection. Gene expression was compared following a shift from 30°C (representing an environment of tropical soil) to either 37°C (representing the environment in a mammalian host) or to 48°C (representing the thermogenic environment of compost) (Nierman et al., 2005). Two interesting clusters of genes showed higher expression levels at 48°C than at 37°C and were enriched for heat shock responsive genes, suggesting that they are integral to thermotolerant growth. A third group had the opposite pattern, with higher expression levels observed at 37°C than at 48°C. However, with the exception of one catalase gene, none of the genes that have already been implicated in the pathogenicity of *A. fumigatus* were enriched in the last group. This suggests that host temperature per se may be insufficient to induce virulence-related gene expression in this fungus or, alternatively, that bona fide temperature-regulated virulence genes have yet to be identified in this species. It is also interesting that each of these clusters contained surprisingly few of the genes involved in the general stress response of *Saccharomyces cerevisiae*, suggesting a fundamental difference between *A. fumigatus* and yeast in this well-characterized stress response. A related study has used microarray analysis to profile temperature-regulated gene expression following a shift from 30 to 37°C (Bhabhra et al., 2008). However, in this case the focus was on a comparison between wild-type *A. fumigatus* and a ribosome biogenesis mutant, in order to identify compensatory pathways related to the process of ribosome synthesis. The data highlighted a cluster of translational machinery mRNAs that were upregulated in the mutant relative to the wild type, thereby providing a rational basis for further analysis of these genes based on the possibility that they may encode proteins that compensate for this ribosome biogenesis defect. The challenge for future work on thermotolerance will be to determine which temperature-regulated genes are important for the growth of *A. fumigatus* at 37°C, so that novel therapeutic strategies can be developed to block their function and reduce the growth of the fungus at mammalian body temperature.

Transcriptional Profiling: Response to Voriconazole

Antifungal drugs exert considerable stress on the fungus when they are used to combat infection. Thus, a comprehensive understanding of how the organism adjusts its physiology in response to antifungals is essential to maximize the therapeutic efficacy of current and future antifungal therapy. Voriconazole is an azole commonly used for the treatment of aspergillosis, which blocks the ergosterol biosynthetic pathway (Ferreira et al., 2005). To gain insight into how *A. fumigatus* responds to voriconazole, a transcriptional profile of *A. fumigatus* hyphae was obtained following exposure to voriconazole for 30, 60, and 240 min (da Silva Ferreira et al., 2006b). A total of 2,271 genes were differentially expressed, emphasizing the profound effect that this drug has on global gene expression. Approximately one-half of the genes involved in *A. fumigatus* ergosterol synthesis were modulated in response to voriconazole, with one cluster showing increased expression and another cluster showing reduced expression. These changes are likely to affect the composition and fluidity of the membrane and thus influence azole susceptibility. Interestingly, the results from this study contrast with the more global upregulation of ergosterol biosynthetic genes that was reported in azole-treated yeast (Bammert and Fostel, 2000; De Backer et al., 2001), suggesting that there may be fundamental differences between yeast and *A. fumigatus* in this regard. Increased expression of various transporters, transcription factors, and proteins involved in metabolism was also identified in voriconazole-treated *A. fumigatus* cultures, although the

contribution of these genes to azole susceptibility awaits further investigation (da Silva Ferreira et al., 2006b). In addition, the expression of several voriconazole-induced mRNAs was shown to require an intact cyclic AMP-protein kinase A (PKA) pathway for full induction (da Silva Ferreira et al., 2006b), suggesting an important role for this signaling pathway in the adaptive response to azole-induced stress.

Identification of Transcription Factor Targets

The ability of high-density microarrays to profile gene expression on a global scale makes them particularly useful to identify targets of transcriptional regulatory proteins. Two examples of this approach have been reported for *A. fumigatus*, focusing on the transcription factor StuA and the protein methyltransferase LaeA. StuA is a basic-helix-loop-helix-containing transcription factor of the APSES family that regulates morphogenesis in ascomycetes (Wu and Miller, 1997). The *A. fumigatus stuA* gene is upregulated after the acquisition of developmental competence, and a Δ*stuA* mutant is defective in conidiation (Sheppard et al., 2005). A comparison of the transcriptional profiles of wild-type *A. fumigatus* with that of the Δ*stuA* mutant identified a distinct cluster of genes that showed StuA-dependent expression, including some encoding enzymes required for secondary metabolites, proteins involved in morphogenesis, and certain allergens (Sheppard et al., 2005). The identification of these StuA-regulated genes provides a foundation for dissecting the poorly understood mechanisms of developmental competence and how competence is linked to conidiation and other essential pathways.

Like other members of its genus, *A. fumigatus* secretes a diverse array of secondary metabolites into its environment (Keller et al., 2005). These chemicals are thought to benefit the fungus by protecting it from competing or predatory species (Gardiner et al., 2005; Rohlfs et al., 2007). However, they may also be secreted in the host during infection, and there is evidence that this might influence disease outcome (Lewis et al., 2005a, 2005b; Sugui et al., 2007b). The biosynthetic enzymes involved in secondary metabolism are often arranged in contiguous clusters in the genome, and the nuclear protein LaeA has an important role in regulating the expression of these clusters (Bok and Keller, 2004). Since LaeA is a predicted protein methyltransferase, its activity as a transcriptional regulator has been hypothesized to be at the level of chromatin modification (Keller et al., 2005). To identify genes that are subject to this epigenetic regulation, a comparison was made between the transcriptional profile of wild-type *A. fumigatus* and that of a Δ*laeA* mutant. The results demonstrated that LaeA has an impressive influence on gene expression in *A. fumigatus*, affecting at least 9.5% of the total genome (Perrin et al., 2007). In addition, the protein was shown to positively regulate 20 to 40% of major classes of secondary metabolite biosynthesis genes (Perrin et al., 2007).

MOLECULAR TOOLS: PROTEOMICS IN *A. FUMIGATUS*

Proteomics combined with mass spectrometry (MS) analysis provides a powerful technique to simultaneously identify multiple gene products that are modulated in response to a changing environment. One of the first studies using this methodology applied to *A. fumigatus* entailed a biochemical approach to release and purify glycosylphosphatidylinositol (GPI)-anchored proteins from *A. fumigatus* membrane preparations, resulting in the resolution of nine GPI-anchored proteins by two-dimensional gel electrophoresis (2DGE) in combination with MS (Bruneau et al., 2001). The 2DGE protocol was subsequently optimized for use in *A. fumigatus* (Kniemeyer et al., 2006) and has been combined with other approaches to gain unique insight into the *A. fumigatus* proteome. For example, *A. fumigatus* is associated with allergic hypersensitivity reactions, ranging in severity from a mild allergy to life-threatening ABPA (Gibson, 2006). In an attempt to identify novel *A. fumigatus* allergens that are clinically relevant, 2DGE was used with immunoblotting, utilizing sera from *A. fumigatus*-sensitized asthmatics or patients with ABPA (Gautam et al., 2007). Subsequent identification of the immunoreactive proteins by matrix-assisted laser desorption ionization–time of flight revealed a total of 16 allergens, the majority of which were newly identified. These candidate allergens may have future diagnostic application in patients with allergic aspergillosis and could lead to the development of novel immunotherapeutics for patients with severe allergy.

A more targeted proteomics strategy has been used to uncover novel cellulases in the *A. fumigatus* proteome. The procedure focuses on the identification of β-glucosidase activity in the secreted proteome using a 2D-in-gel β-glucosidase activity assay combined with tandem MS (Kim et al., 2007). This approach led to the identification of two novel secreted β-glucosidases, validating the utility of this in situ activity staining as a way to assess the enzymatic activity of the *A. fumigatus* secreted proteome. Interestingly, one of two *A. fumigatus* β-glucosidases identified in this screen revealed striking heat stability compared to the previously characterized β-glucosidases from other aspergilli. The thermostability of this enzyme reflects the unique thermotolerance of *A. fumigatus* and may have industrial application in cellulase systems (Kim et al., 2007).

The killing of *A. fumigatus* by the innate immune system is mediated, at least in part, by the generation of toxic reactive oxygen intermediates (Chauhan et al., 2006). In order to understand the reactive oxygen intermediate detoxifying systems in the fungus, a 2DGE approach followed by matrix-assisted laser desorption ionization–time of flight was used to examine the response of *A. fumigatus* hyphae to hydrogen peroxide (Lessing et al., 2007). The results identified a number of candidate genes, one of which was the homologue of the well-known regulator of yeast oxidative stress responses, Yap1. A second proteomic comparison was then employed to identify candidate Yap1 target genes, one of which was identified as catalase 2. This study represents one of the most comprehensive applications of proteomics to the biology of *A. fumigatus*, since it integrated two proteomic analyses with a genetic approach to understand the function of a candidate target gene.

MOLECULAR TOOLS: ANALYSIS OF THE *A. FUMIGATUS* GENOME

The genomic sequence of *A. fumigatus* isolate Af293 was published in 2005 (Nierman et al., 2005). This landmark paper provided the first insight into the remarkable physiology of this fungus and revealed some surprising discoveries. The Af293 genome was sequenced using a whole-genome random shotgun sequencing method combined with optical mapping. The size of the genome was initially reported as 29.4 Mb spanning eight chromosomes, but this was subsequently adjusted to 28.8 Mb (Fedorova et al., 2008).

Evidence for PCD Pathways

Programmed cell death (PCD) is an important stress response among metazoans, and components of the pathway are considered to be excellent targets for anticancer drug development. By analogy to the cancer field, fungal PCD pathways would also be expected to have merit as antifungal drug targets. The potential for manipulating PCD in pathogenic fungi is supported by the discovery of apoptosis-like death in both yeast and filamentous fungi (Eisenberg et al., 2007; Ramsdale, 2008). Although the *A. fumigatus* genome lacks the upstream regulators of the metazoan apoptotic machinery, it contains some of the downstream effectors that have been implicated in mammalian apoptosis (Fedorova et al., 2005; Nierman et al., 2005). However, since many components of the PCD machinery in mammals have prosurvival functions in addition to their roles in cell death, the existence of these proteins in lower eukaryotes does not necessarily indicate a role in PCD signaling (Garrido and Kroemer, 2004; Schwerk and Schulze-Osthoff, 2003). Ultrastructural and biochemical changes that are characteristic of apoptosis have been reported in *A. fumigatus* (Mousavi and Robson, 2003, 2004). However, current evidence indicates that *A. fumigatus* caspase-like proteins are dispensable for death induced by multiple adverse environmental conditions (Richie et al., 2007b). This raises the possibility that there may be redundant pathways of PCD that can be activated under these conditions or, alternatively, that metacaspases are not key players in fungal PCD.

In higher eukaryotes, prolonged endoplasmic reticulum (ER) stress activates caspase-dependent apoptosis. This is in striking contrast to *A. fumigatus*, where the metacaspases have a protective role under conditions of ER stress, rather than a death-promoting function (Richie et al., 2007b). One possible explanation for this difference is that fungal metacaspases provide a benefit to the organism under conditions of ER stress, such as growth on a complex substrate that requires a high secretory load. With the advent of multicellularity, this homeostatic mechanism may have diverged into PCD, which also provides a benefit to an organism by eliminating cells that have become irreversibly damaged by unresolved ER stress.

Evidence for a Highly Secretory Lifestyle

In order to thrive in the environment, *A. fumigatus* must continually extract nutrients from decaying vegetation. This requires an ability to secrete copious quantities of degradative enzymes. A large and diverse array of secreted hydrolases are predicted in the *A. fumigatus* genome (Robson et al., 2005), including at least 99 secreted proteases (Machida et al., 2005; Nierman et al., 2005), a feature that reflects the ecological niche of the fungus in decaying organic material. However, it appears that *A. fumigatus* lacks the lignin-degrading machinery that allows fungi such as *Phanerochaete chrysosporium* to grow on this complex substrate (Tekaia and Latge, 2005).

Evidence for a Sexual Cycle

Although *A. fumigatus* has no identified sexual cycle, genome analysis has revealed the existence of genes involved in the mating process, pathway signaling, fruiting body development, and meiosis, suggesting that *A. fumigatus* has an extant sexual cycle that remains to be observed (Poggeler, 2002; Varga, 2003). In heterothallic ascomycetes, mating type is determined by genes at a single *MAT* locus. Opposite mating-type loci do not show any sequence similarity, even if they occupy the same chromosomal region, so they are referred to as idiomorphs. Sequence analysis revealed that the *MAT* locus of *A. fumigatus* contains highly conserved sequences flanking an idiomorphic region that shows little homology between two types of isolates: a *MAT1-1* idiomorph contains a single open reading frame (ORF) encoding the α-box gene, and a *MAT1-2* idiomorph contains a single ORF encoding a high-mobility group (HMG) gene. This idiomorph structure is characteristic of sexually reproducing heterothallic ascomycete fungi (Paoletti et al., 2005). A heterothallic mating system would be expected to facilitate variation and thus promote adaptation to dynamic environmental conditions. Consistent with this possibility, analysis of 290 worldwide clinical and environmental isolates revealed the existence of *MAT1-1* and *MAT1-2* genotypes of *A. fumigatus* in approximately equivalent proportions (Paoletti et al., 2005). Interestingly, the *MAT1-1* locus was also found to contain a 360-bp fragment of an HMG gene neighboring the idiomorphic region. Based on this finding, it has been proposed that *A. fumigatus* arose from a homothallic ancestor in which α- and HMG genes were adjacent, but the genes subsequently became separated as a result of gene loss (Galagan et al., 2005). The expression of mating-type, pheromone precursor, and pheromone receptor genes can be detected in *A. fumigatus*, and population genetic studies have provided evidence that recombination is occurring within populations in the environment (Paoletti et al., 2005).

The functionality of the *A. fumigatus* Mat1-2 protein was recently examined by an interspecies gene exchange between *A. fumigatus* and *Aspergillus nidulans* (Pyrzak et al., 2008). When driven by its own promoter, an ectopically integrated *A. fumigatus mat1-2* gene was not able to complement the lack of sexual development in a Δ*matA* mutant of *A. nidulans*. In contrast, when the *A. fumigatus mat1-2* gene was placed under the control of the *A. nidulans matA* promoter and integrated into the native *matA* locus, it was able to confer full fertility upon the Δ*matA* mutant of *A. nidulans*. These data demonstrate functionality of the *A. fumigatus* Mat1-2 protein and raise the possibility that expression levels are central to the fertility of this species. Taken together, these data are consistent with a recent history of sexual recombination, raising the possibility that sexual activity is still ongoing in *A. fumigatus*. Since the existence of a sexual cycle has important implications for our understanding of the biology and virulence of this species, the challenge for the future will be to determine if a complete sexual cycle of *A. fumigatus* can be induced in the laboratory.

Identification of Potential Virulence Genes by Genome Comparisons

Since environmental prevalence alone does not predict the association of *A. fumigatus* with human infection, it is likely that *A. fumigatus* has certain virulence attributes that set it apart from other less pathogenic environmental molds (Hospenthal et al., 1998). Thus, a major goal of comparative genomic analysis is to identify *A. fumigatus*-specific genes, with the assumption that these genes evolved to facilitate rapid niche adaptation in the environment and may, therefore, be relevant to adaptation to the host. A comparison of the *A. fumigatus* genome with that of *A. oryzae* and *A. nidulans* reveals approximately 500 putative *A. fumigatus*-specific genes (Nierman et al., 2005). However, their contribution to the biology of *A. fumigatus* is complicated by the significant phylogenetic distance between these species. The three genomes vary in size from 29 to 37 Mb, with *A. fumigatus* being the smallest. Despite being members of the same genus, predicted orthologues from these species share a level of amino acid identity that

is comparable to that observed between mammals and fish, which are separated by ~450 million years (Fedorova et al., 2008; Galagan et al., 2005). This may reflect accelerated divergence within the genus, rather than an ancient separation (Cai et al., 2006). One example of the evolution of niche-specific adaptation is the presence of a complement of genes involved in arsenic metabolism, some of which have only bacterial or archaeal homologues (Nierman et al., 2005).

By aligning three-way orthologous genes, including 1 kb of flanking sequence, blocks of striking conservation were identified outside the protein-coding regions in *A. fumigatus*, *A. oryzae*, and *A. nidulans* (Galagan et al., 2005). A list of high-scoring conserved sequences was generated by applying a conservation scoring function, and a significant proportion of the high-scoring conserved sequences were predicted to lie within transcribed, but untranslated regions. Short, upstream ORFs (uORFs) are an important translational control element located in the 5′ untranslated region and are frequently involved in pathways that control cell growth, particularly under conditions of environmental stress (Vilela and McCarthy, 2003). For this reason, translational regulation by uORFs is often found in mRNAs that encode proteins with functions in the adaptive response to environmental stress (Hoffmann et al., 2001; van den Brink et al., 2000; Vilela et al., 1998; Vilela and McCarthy, 2003). The mechanisms by which uORFs regulate the expression of the main ORF in response to changes in environmental conditions include regulating the rate of reinitiation of the ribosome on the main ORF, producing a *cis*-acting peptide that stalls the ribosome on the uORF, or destabilizing the mRNA (Vilela and McCarthy, 2003). The extent to which uORF-mediated translational regulation contributes to fungal virulence is not yet known. However, a recent investigation of the genomic distribution and conservation of uORFs among strains of the basidiomycete *Cryptococcus neoformans* has suggested that uORFs are important and widespread elements of posttranscriptional regulation that promote the fitness of this fungus and may, therefore, contribute to virulence (Neafsey and Galagan, 2007). Genome comparisons between *A. fumigatus*, *A. nidulans*, and *A. oryzae* suggest that translational regulation by uORFs is more common among the filamentous fungi than in *S. cerevisiae* (Galagan et al., 2005; McCarthy, 1998). At least one established virulence determinant in *A. fumigatus*, the cross-pathway control transcription factor CpcA (Krappmann et al., 2004), is subject to uORF-mediated translational regulation (Hoffmann et al., 2001), suggesting that the presence of uORFs in *A. fumigatus* may indeed be relevant to the virulence of this organism.

To gain further insight into how the genome of *A. fumigatus* contributes to pathogenesis, a second clinical isolate was sequenced, A1163 (Fedorova et al., 2008). The genome of A1163 is 1.4% larger than the genome of Af293, and a comparison of the two isolates revealed the presence of ~2% unique genes. Interestingly, more than one-half of the Af293-specific genes are also absent in *A. fumigatus* isolates Af294 and Af71 by array-based comparative genome hybridization, raising the possibility that they represent genes that evolved to confer niche-specific adaptability in the environment (Fedorova et al., 2008). In keeping with this notion, one-fifth of the Af293-specific genes appear to have arisen as a consequence of two segmental duplication events, including an arsenic detoxification cluster and genes that may be involved in betaine metabolism. The absence of these duplication events in the Af294 and Af71 isolates suggests that they occurred relatively recently, a finding that is consistent with the idea that segmental duplication events may be involved in rapid adaptation.

The majority of the genes in Af293 and A1163 represent core genes that are 99.8% identical at the nucleotide level. However, several variable loci can be identified with levels of identity as low as 40%, the most divergent of which appear to contain heterokaryon incompatibility (HI) genes (*het* genes). HI is a type of nonself recognition that triggers PCD when fungal hyphae of unlike genotype fuse (Glass and Dementhon, 2006; Pinan-Lucarre et al., 2003). Although HI has yet to be studied experimentally in *A. fumigatus*, doing so would be a worthwhile endeavor, because HI is thought to be ubiquitous among filamentous ascomycetes. The identification of *het* gene diversity in *A. fumigatus* isolates opens the way for future studies into possible strategies to manipulate PCD for therapeutic gain.

Cross-species comparisons between *A. fumigatus*, *Neosartorya fischeri*, and *A. clavatus* revealed that 8.5% of the genes in *A. fumigatus* are specific to that species. Members of the *A. fumigatus*-specific group are small, with few introns, and tend to cluster in subtelomeric chromosomal islands that are enriched for pseudogenes, transposons, and repetitive elements. Analysis of gene ontology terms associated with these *A. fumigatus*-specific genes revealed diverse functions in carbohydrate and amino acid metabolism, transport, detoxification, and secondary metabolite synthesis. Since most of the currently identified virulence-associated genes of *A. fumigatus* (Table 1) show a low rate of protein evolution between species (Fedorova et al., 2008), the genes located in these islands may provide a rich source of novel genes that influence niche-specific adaptability and thus virulence.

MOLECULAR TOOLS: GENETIC MANIPULATION OF *A. FUMIGATUS*

Genetic Screens

The genetic tractability of *A. fumigatus* has improved tremendously over the past decade, resulting in a steady rise in the number of publications that use molecular tools to probe *A. fumigatus* biology and virulence (Krappmann, 2006). Despite the success of genetic screens in other fungal systems, their application to *A. fumigatus* has thus far been limited. One notable exception is a signature-tagged mutagenesis strategy that was employed to identify genes that are essential for virulence in a mouse infection model (Brown et al., 2000). Random insertion mutants were used infect mice in mixed infection experiments, resulting in the identification of *p*-aminobenzoic acid (PABA) synthase as a gene that is required for virulence. However, no further studies using this technique have been reported.

Selectable Markers

Several isogenic auxotrophic mutant strains of *A. fumigatus* have been developed so that transformants can be selected for by complementation of nutritional requirements (Weidner et al., 1998; Xue et al., 2004a). In addition, three major resistance cassettes have been successfully used for selection in a wild-type background: the *Escherichia coli hph* gene, encoding resistance to hygromycin; the *Streptoalloteichus hindustanus ble* gene, encoding resistance to phleomycin; and the *A. oryzae ptrA* allele, conferring resistance to pyrithiamine. A recyclable marker system is also available, which enables positive and negative selection in a wild-type background (Krappmann et al., 2005). Recycling of the cassette is accomplished by expressing a fusion between the phleomycin

TABLE 1 Virulence of gene deletion mutants of *A. fumigatus*[a]

Functional category	Disruption	Model	Virulence	Reference(s)
Antioxidant defense				
Catalase	Δ*catA*	Rat (it)	Reduced (Δ*cat1*/Δ*cat2*)	Paris et al., 2003b
	Δ*cat1*/Δ*cat2*			
Catalase	Δ*cat1*	Mo (in)	WT	Calera et al., 1997
Nonribosomal peptide synthetase	Δ*pes1*	Gm	Reduced	Reeves et al., 2006
Transcription factor	Δ*yap1*	Mo (in)	WT	Lessing et al., 2007
Transcription factor	Δ*skn7*	Mo (in)	WT	Lamarre et al., 2007
Cell wall and membrane				
Chitin synthase	Δ*chsC*	Mo (in)	Reduced	Mellado et al., 1996a
	Δ*chsG*		(Δ*chsG*/*chs*C)	
	Δ*chsG*/*chs*C			
Chitin synthase	Δ*chsD*	Mo (in)	WT	Mellado et al., 1996b
Chitin synthase	Δ*chsE*	Mo (in)	WT	Aufauvre-Brown et al., 1997
Chitinase	Δ*chiB1*	NR	NR	Jaques et al., 2003
Conidial hydrophobin	Δ*rodA*	Mo (in)	WT	Thau et al., 1994
Conidial hydrophobin	Δ*rodB*	NR	NR	Paris et al., 2003a
GPI-*N*-acetylglucosaminyltransferase	Δ*pig-a*	Mo (in)	Reduced	Li et al., 2007
β(1-3)Glucanosyltransferase	Δ*gel1*	Mo (in)	Reduced (Δ*gel2*)	Mouyna et al., 2005
	Δ*gel2*		Reduced (Δ*gel1*/Δ*gel2*)	
	Δ*gel1*/Δ*gel2*			
GPI-anchored protein	Δ*ecm33*	Mo (iv)	Increased	Romano et al., 2006
UDP-galactopyranose mutase	Δ*glfA*	Mo (in)	Reduced	Schmalhorst et al., 2008
α(1-3)Glucan synthase	Δ*ags1*	Mo (in)	WT (Δ*ags1*, Δ*ags2*)	Beauvais et al., 2005
	Δ*ags2*			
α(1-3)Glucan synthase	Δ*ags3*	Mo (in)	Increased	Maubon et al., 2006
O-Mannosyltransferase	Δ*pmt1*	Mo (in)	WT	Zhou et al., 2007
β(1-3)Endoglucanase	Δ*engl1*	NR	NR	Mouyna et al., 2002
Polyketide synthase (pigment)	Δ*alb1* (Δ*pksP*)	Mo (iv)	Reduced	Langfelder et al., 1998; Tsai et al., 1998
Laccase (pigment)	Δ*abr2*	Mo (in)	WT	Sugareva et al., 2006
Scytalone dehydratase (pigment)	Δ*arp1*	NR	NR	Tsai et al., 1999
C-5 sterol desaturase	Δ*erg3A*	NR	NR	Alcazar-Fuoli et al., 2006
	Δ*erg3B*			
	Δ*erg3A*/Δ*erg3B*			
14α-Demethylase	Δ*cyp51A*	Mo (in)	WT	Mellado et al., 2005
Signaling pathways				
Oxylipin synthesis: dioxygenases	*ppo*ABC(*RNAi*)	Mo (in)	Increased	Tsitsigiannis et al., 2005
Oxylipin synthesis: dioxygenases	Δ*ppoA*	Mo (in)	WT (Δ*ppoC*)	Dagenais et al., 2008
	Δ*ppoB*		NR (Δ*ppoA*, Δ*ppoB*)	
	Δ*ppoC*			
Two-component signaling: histidine kinase Fos-1	Δ*fos-1*	Mo (iv)	Reduced	Clemons et al., 2002
PKA signaling: PKA catalytic subunit C1	Δ*pkaC1*	Mo (in)	Reduced	Liebmann et al., 2004b
G protein α subunit B	Δ*gpaB*			
PKA signaling: G protein α subunit B	Δ*gpaB*	NR	NR	Liebmann et al., 2003
adenylate cyclase	Δ*acyA*			
PKA signaling: PKA regulatory subunit	Δ*pkaR*	Mo (in)	Reduced	Zhao et al., 2006
Ras signaling: RasB	Δ*rasB*	Mo (in)	Reduced	Fortwendel et al., 2005

Functional category	Disruption	Model	Virulence	Reference(s)
Ras signaling: RasA and RasB	*ras*A(G17V)[da] *ras*B(G16V)[da] *ras*A(S22N)[dn] *ras*B(T21N)[dn]	NR	NR	Fortwendel et al., 2004
Calcineurin signaling: catalytic subunit CnaA	Δ*cnaA*	Mo (in, inh, iv) *Gm*	Reduced	da Silva Ferreira et al., 2007; Steinbach et al., 2006
Calcineurin signaling: transcription factor CrzA	Δ*crzA*	Mo (in)	Reduced	Cramer et al., 2008; Soriani et al., 2008
TOR/RHEB signaling: RhbA	Δ*rhbA*	Mo (in)	Reduced	Panepinto et al., 2003
MAPK signaling: Sho1 adaptor (Hog1 pathway)	Δ*sho1*	Mo (in)	WT	Ma et al., 2008
MAPK signaling: MpkA	Δ*mpkA*	Mo (in)	WT	Valiante et al., 2008
MAPK signaling: SakA (Hog1)	Δ*sakA*	NR	NR	Xue et al., 2004b
MAPK signaling: MpkC	Δ*mpkC*	NR	NR	Reyes et al., 2006
MAP kinase signaling: SakA (Hog1)	Δ*sakA*	NR	NR	Du et al., 2006
TcsB (sensor histidine kinase)	Δ*tcsB*			
ER stress: metacaspases	Δ*casA* Δ*casB* Δ*casA*/Δ*casB*	Mo (in)	WT (Δ*casA*/Δ*casB*)	Richie et al., 2007b
Asexual development	Δ*flbA* Δ*brlA* Δ*fluG* *gpa*A(Q204L)[da] *gpa*A(G203R)[dn]	NR	NR	Coyle et al., 2007; Mah and Yu, 2006
Asexual development	Δ*stuA*	Mo (in)	WT	Sheppard et al., 2005
Secreted products				
Secondary metabolites: protein methyltransferase regulating secondary metabolite clusters	Δ*laeA*	Mo (in)	Reduced	Bok et al., 2005; Sugui et al., 2007a
Secondary metabolites: transcription factor regulating secondary metabolite expression	Δ*gliZ*	Mo (in)	WT	Bok et al., 2006
Gliotoxin: nonribosomal peptide synthase	Δ*gliP*	Mo (in/ih)	Reduced (Sugui) WT (Kupfahl) WT (Cramer) Reduced (Spikes)	Cramer et al., 2006; Kupfahl et al., 2006; Spikes et al., 2008; Sugui et al., 2007b
Unknown: nonribosomal peptide synthase	Δ*pes1*	*Gm*	Reduced	Reeves et al., 2006
Proteases: alkaline protease	Δ*alp*	Mo (in/ih)	WT	Monod et al., 1993; Tang et al., 1993
Proteases: alkaline protease metalloprotease	Δ*alp*/Δ*mep*	Mo (in)	WT	Jaton-Ogay et al., 1994
Proteases: serine protease	Δ*alp2*	NR	NR	Reichard et al., 2000a
Proteases: aspergillopepsin	Δ*pep*	Gp (iv) Mo (in)	WT	Reichard et al., 1997
Proteases: aspartic proteinase	Δ*pep2*	NR	NR	Reichard et al., 2000b
Toxins: restrictocin	Δ*res*	Mo (in)	WT	Paris et al., 1993; Smith et al., 1993
Proteases and toxins: alkaline protease restrictocin	Δ*alp*/Δ*res*	Mo (in)	Reduced	Smith et al., 1994
Nutritional requirements				
Iron: siderophore biosynthesis	Δ*sidA* Δ*sidC* Δ*sidD* Δ*sidF* Δ*sidG*	Mo (in)	Reduced (Δ*sidA*, Δ*sidC*, Δ*sidD*, Δ*sidF*) WT (Δ*sidG*)	Hissen et al., 2005; Schrettl et al., 2004; Schrettl et al., 2007

(Continued on next page)

TABLE 1 Virulence of gene deletion mutants of *A. fumigatus*[a] (*Continued*)

Functional category	Disruption	Model	Virulence	Reference(s)
Iron: reductive Fe assimilation	Δ*ftrA*	Mo (in)	WT	Schrettl et al., 2004
Iron: siderophore degradation	Δ*estB*	NR	NR	Kragl et al., 2007
Zinc: zinc transporters	Δ*zrfA*	NR	NR	Vicentefranqueira et al., 2005
	Δ*zrfB*			
	Δ*zrfA*/Δ*zrfB*			
Zinc: zinc-responsive transcription factor	Δ*zafA*	Mo (in)	Reduced	Moreno et al., 2007
Amino acid homeostasis: homoaconitase (lysine)	Δ*lysF*	Mo (in)	Reduced	Liebmann et al., 2004a
Folate biosynthesis: PABA synthetase	Δ*pabaA*	Mo (in)	Reduced	Brown et al., 2000; Sandhu et al., 1976
Pyrimidine biosynthesis: orotidine-5′-decarboxylase	Δ*pyrG*	Mo (in)	Reduced	d'Enfert et al., 1996
Glyoxylate cycle: isocitrate lyase	Δ*acuD*	Mo (in)	WT	Schobel et al., 2007
Glyoxylate cycle: isocitrate lyase and malate synthase	Δ*acuD*	Mo (in)	WT (Δ*acuD*, Δ*acuE*)	Olivas et al., 2008
	Δ*acuE*			
Methyl citrate cycle: methyl citrate synthase	Δ*mcsA*	*Gm* Mo (in)	Reduced	Ibrahim-Granet et al., 2008; Maerker et al., 2005
Nitrogen utilization: transcription factor	Δ*areA*	Mo (in)	Reduced	Hensel et al., 1998
Amino acid homeostasis: cross pathway control system	Δ*cpcA*	Mo (in)	Reduced	Krappmann et al., 2004
Amino acid homeostasis: cross pathway control induction	Δ*cpcC*	Mo (in)	WT	Sasse et al., 2008
Autophagy: Atg1 serine kinase	Δ*atg1*	Mo (it)	WT	Richie et al., 2007a
Thermotolerance				
Unknown	Δ*thtA*	Mo (in)	WT	Chang et al., 2004
Ribosome biogenesis	Δ*cgrA*	Mo (in) *Dm*	Reduced	Bhabhra et al., 2004
Mutants generated for methodology purposes				
ABC family transporter	Δ*abcA*	NR	NR	Langfelder et al., 2002
Homoisocitrate dehydrogenase	Δ*lysB*	NR		Xue et al., 2004a
Ornithine transcarbamylase	Δ*argB*	NR	NR	Jadoun et al., 2004; Xue et al., 2004a
Homoisocitrate dehydrogenase	Δ*lysB*			
Ku70	Δ*akuA*	*Gm*	WT (Δ*akuA*)	Krappmann et al., 2006
	Δ*abr2*			
Ku80	Δ*akuB*	Mo (in)	WT	da Silva Ferreira et al., 2006a
PABA synthase	Δ*pabaA*	NR	NR	Krappmann et al., 2005
VeA	Δ*veA*			

[a]Abbreviations: Mo, mouse; *Gm*, *G. mellonella*; Gp, guinea pig; *Dm*, *D. melanogaster*; in, intranasal; it, intratracheal; iv, intravenous; inh, inhalational; WT, wild type; NR, not reported.

resistance gene and thymidine kinase, allowing for positive selection in the presence of phleomycin and negative selection in the presence of nucleoside analogs. In addition, the system employs flanking *loxP* sites, enabling marker excision by transient expression of Cre recombinase.

Gene Disruption

The ability to completely ablate the expression of a gene by targeted deletion remains one of the most powerful approaches to understanding gene function in *A. fumigatus*. Gene deletion is most often accomplished by creating a deletion construct containing a selectable marker that is flanked by at least 1 kb of homology to the gene of interest. An increase in gene targeting rate can sometimes be accomplished by transforming with two overlapping fragments of a deletion cassette rather than using a single deletion fragment. Improvements in homologous targeting frequencies have also been reported using recipient strains that have been deleted for subunits of the Ku heterodimer required for nonhomologous end joining (NHEJ) (da Silva Ferreira et al., 2006a; Krappmann et al., 2006). The frequency of homologous targeting in the Δ*akuA* or Δ*akuB* mutants is considerably higher than in wild-type *A. fumigatus*, and the length of the flanking arms that are necessary for homologous recombination is much less, making these strains particularly useful for high-throughput strategies of gene

deletion. Importantly, the two NHEJ mutant strains are indistinguishable from the wild type on plates and do not show any evidence of gross alterations in virulence in mouse models of aspergillosis. However, since a defect in NHEJ could potentially influence the phenotype of certain deletion mutants, this caveat should always be kept in mind when using Δ*akuA* or Δ*akuB* genetic backgrounds as a basis for further genetic manipulation.

Regulation of Gene Expression by Promoter Replacement

An alternative approach to gene deletion is to perform a promoter replacement using a promoter that can be experimentally manipulated. Several regulatable promoters have been used in *A. fumigatus*, including the alcohol-inducible promoter *alcA* (Romero et al., 2003), the C-2 carbon source-inducing promoter *acuD* (Grosse et al., 2008), the carboxymethylcellulose-inducible *cbhB* promoter (Bromley et al., 2006; Khalaj et al., 2007), and the doxycycline-regulated promoter *tetO* (Vogt et al., 2005). However, the most extensive application of the inducible promoters in *A. fumigatus* has been the use of the nitrogen-regulated promoter *niiA*, in which a conditional promoter replacement strategy was exploited for essential gene identification and drug target prioritization (Hu et al., 2007). This approach demonstrated the essentiality of 35 *A. fumigatus* genes from a set of 54 genes whose orthologues were known to be essential in *Candida albicans* and *S. cerevisiae*. The study was the first to incorporate a systematic conditional promoter replacement into a high-throughput screen for essential genes in *A. fumigatus* and will have important application in the search for novel antifungal inhibitors of essential gene function. Inducible promoters sometimes suffer from lack of tight regulation, which may or may not be a concern depending on the specific application. In addition, they become technically problematic if the condition required to induce the promoter also influences the activity of the pathway that is under investigation. Despite these caveats, it is now clear that inducible promoters can be used to modulate gene expression in *A. fumigatus* and will serve as invaluable tools in future studies of gene function and virulence in this organism.

Regulation of Gene Expression by RNAi

Downregulation of gene expression by RNA interference (RNAi) is now a widespread tool for manipulating gene expression in multiple species and has been successfully used to downregulate gene expression in *A. fumigatus* (Bromley et al., 2006; Henry et al., 2007; Khalaj et al., 2007; Mouyna et al., 2004; Tsitsigiannis et al., 2005). Despite these encouraging results, problems with incomplete silencing and instability have been reported (Henry et al., 2007). In view of the off-target effects that have been reported for RNAi in other systems (Svoboda, 2007), several hurdles need to be overcome before this technique can replace classical gene disruption and deletion in *A. fumigatus*.

VIRULENCE IN *A. FUMIGATUS*

A typical primary pathogen has unique virulence traits that evolved in association with a host organism. These virulence factors are generally dispensable for growth outside the host but provide some competitive advantage to the organism when it is in the host environment. For an opportunistic saprophytic fungus like *A. fumigatus*, factors that influence growth in the host are thought to overlap with those that have evolved to enhance competitiveness in the natural environment of decaying vegetation. Thus, many of the genes that have been shown to influence pathogenicity in *A. fumigatus* do not fit neatly into a classical definition of a virulence factor. However, their contribution to virulence provides important insight into how *A. fumigatus* adjusts its physiology in the host environment, which has the potential to generate novel approaches to disrupt this adaptability.

Virulence-Related Genes of *A. fumigatus*

The candidate gene approach has been used extensively to gain insight into genes that influence the virulence of *A. fumigatus*. Most of these mutants were generated by gene deletion or disruption using homologous targeting of selectable markers, although examples of gene manipulation by RNAi and the expression of dominant negative and dominant active alleles have also been reported (Fortwendel et al., 2004; Mah and Yu, 2006; Tsitsigiannis et al., 2005). The mutants of *A. fumigatus* that have been generated to date are listed in Table 1 and are clustered by functional category of the mutated genes. The functions of these genes are diverse, including antioxidant defense, cell wall or membrane integrity, intracellular signaling in response to external cues, nutrient acquisition, thermotolerance, and the secretion of damaging products, such as secondary metabolites, proteases, and toxins. Approximately 60% of the mutants that have been tested in animal models show altered virulence. Although changes in virulence in animal models often correlate with an alteration in growth rate, some notable exceptions suggest that the link between growth rate and virulence is not absolute and should be considered as only part of the virulence phenotype. For example, deletion of *cnaA*, encoding the catalytic A subunit of calcineurin, generated a severely growth-impaired mutant that was incapable of causing disease in animal models (da Silva Ferreira et al., 2007; Steinbach et al., 2006). Strikingly, a mutant lacking the calcineurin-dependent transcription factor CrzA grew at a rate similar to that of the wild type in vitro but was still hypovirulent (Soriani et al., 2008). A second example of this is provided by mutants of the mitogen-activated protein kinase (MAPK) pathway. Despite a considerable growth defect on standard medium, a Δ*mpkA* mutant was as virulent as wild-type *A. fumigatus* (Valiante et al., 2008). Similarly, deletion of an upstream regulator of the high-osmolarity glycerol (HOG)-MAPK pathway, *sho1*, also impaired in vitro growth rate without affecting virulence (Ma et al., 2008). Further analysis of strains that do not show the usual correlation between in vitro growth rate and virulence could provide valuable insight into mechanisms of pathogenesis.

Finally, it is worthwhile pointing out that the genes listed in Table 1 do not adequately explain why *A. fumigatus* is a more potent opportunistic pathogen than other commonly encountered environmental molds. Perhaps virulence is the result of the activity of multiple gene products acting in concert to facilitate adaptability to the host environment, a notion that poses a significant challenge for future research.

ANIMAL MODELS OF INVASIVE ASPERGILLOSIS

In order to evaluate virulence and other effects on pathogenesis, isogenic sets, which include a wild type, a mutant, and a complemented mutant, must be tested in an animal model of disease. Numerous animal models for invasive aspergillosis have been described in the literature, and some of the relative strengths and weaknesses of them are discussed below.

Invertebrate Models

The use of invertebrate infection models in studies of pathogenic fungi has increased markedly during the last decade (Mylonakis et al., 2007). The benefits of using such models include the low cost of acquisition and maintenance, the freedom from regulation that accompanies vertebrate models, and in the cases of *Drosophila melanogaster*, *Dictyostelium discoideum*, and *Caenorhabditis elegans*, the number of potentially useful, defined mutants available. Two of these invertebrate hosts, the fruit fly, *D. melanogaster*, and the greater waxmoth larva, *Galleria mellonella*, have been used to examine virulence or drug susceptibility in *A. fumigatus* (Bhabhra et al., 2004; Lionakis et al., 2005; Reeves et al., 2006; Renwick et al., 2006). As in humans, hosts with intact innate immunity are not susceptible to invasive aspergillosis, so work with *Drosophila* has employed flies deficient in the *Toll* receptor system of innate immunity (Bellocchio et al., 2004; Bretz et al., 2008; Lionakis et al., 2005). Flies have been inoculated with *A. fumigatus* by directly injecting the inoculum into the thorax with a needle, by feeding the flies food mixed with conidia from the organism, or by rolling the flies in a plate containing the fungus, thereby coating them with conidia (Lionakis et al., 2005). All three methods resulted in significant mortality, but direct inoculation and ingestion appeared to cause higher mortality. Ingestion has also been used for drug delivery; voriconazole was shown to improve the survival of inoculated flies given this method of treatment (Lionakis et al., 2005). The larvae of the greater waxmoth, *G. mellonella*, have also been used for pathogenesis studies of several medically important fungi, including *A. fumigatus*. In this model, the germination state of the infecting conidia was shown to be key (Renwick et al., 2006). When resting wild-type conidia were injected into the hemocoel via the last footpeg, deaths were not observed until inocula of 10^7 conidia were used. However, preincubating the conidia until they began to swell before inoculation resulted in lethal infections with 10-fold fewer conidia. By use of this model, a deletion mutant of the nonribosomal peptide synthetase Pes1 (Table 1) was shown to possess attenuated virulence (Reeves et al., 2006). Although there is more flexibility in mode of inoculation for the fly model, the waxmoth larvae can be incubated at 37°C, which makes the waxmoth model amenable to testing mutants involved in thermotolerance.

Vertebrate Models

Reports of vertebrate animal models of invasive aspergillosis first began appearing in the literature in the 1950s, concomitant with the increased numbers of cases in humans that were being reported (Sidransky and Friedman, 1959). Sidransky and his colleagues published a series of papers on mouse models in which they described an apparatus for delivering conidia via inhalation; the effects of cortisone, antibiotics, malnutrition, X-irradiation, and cytotoxic agents on the susceptibility to invasive pulmonary disease; survival of infection; and pathology of disease (Epstein et al., 1967; Sidransky and Friedman, 1959; Sidransky et al., 1965). Model development and standardization have remained an area of interest, leading the National Institute of Allergy and Infectious Diseases to support the development of the Invasive Aspergillosis Animal Models core facility to produce standard operating procedures for animal testing (http://www.sacmm.org/iaam.html). These protocols are available at their website and provide good foundations for investigators in the field who wish to institute work with animal models in their laboratories. Their murine models and analysis tools have been extensively validated (Sheppard et al., 2006). Mice remain the most commonly used vertebrate animal model, and most immunosuppressive regimens are based on the principles described in these early reports.

Murine Models of Invasive Aspergillosis

Mice, like humans, are intrinsically resistant to invasive disease caused by *Aspergillus fumigatus*. Therefore, mouse models use a combination of genetic backgrounds, immunosuppressive regimens, and inoculum delivery modes in order to produce pulmonary infections that mimic those seen in humans. Outbred mice have been used by numerous investigators, using the argument that the human population is outbred, and for many purposes these mice are adequate for the experiment (Eisenstein et al., 1990; Sidransky and Friedman, 1959; Smith et al., 1994; Steinbach et al., 2004). Much of the research on the immune response to *A. fumigatus* has taken advantage of the defined backgrounds in inbred strains of mice (Balloy et al., 2005; Bonnett et al., 2006; Mehrad et al., 1999; Montagnoli et al., 2006; Stephens-Romero et al., 2005). These studies have contributed significantly to our understanding of the host response to *A. fumigatus* and the interaction between the fungus and host cells in the recognition and eradication of inhaled conidia. For example, the importance of T-regulatory-cell networks in the recognition of conidia, through T-cell regulation of cytokine production and recognition, is dependent upon the use of inbred mouse models (Montagnoli et al., 2006). Likewise, the role of the chemokine system in resistance to aspergillosis, via their influence on effector cells that are elicited by the fungus and its metabolites, was elucidated through the use of inbred mice (Mehrad et al., 1999). In addition to differences in immunoregulatory factors among inbred strains of mice, a recent study has shown that a wide range of susceptibility to invasive aspergillosis can be demonstrated among inbred strains of mice, even when all receive the same type of immunosuppression and inoculum. It is notable that these differences map with nonsynonymous mutations in the plasminogen gene in both mice and humans (Zaas et al., 2008). Finally, mice that have specific gene deletions have served as useful models for studies of invasive aspergillosis. An example of the power of these models is the $gp91^{phox}$ deletion mouse that is used as a model for chronic granulomatous disease (Morgenstern et al., 1997). The lack of a respiratory burst in the neutrophils of these animals resulted in exquisite susceptibility to inhalation of *A. fumigatus* conidia, causing them to develop progressive pulmonary disease without the requirement for further immunosuppression. This is very similar to what is seen in patients suffering from the X-linked form of chronic granulomatous disease.

A variety of immunosuppressive regimens are used to render animals susceptible to aspergillosis. Early studies with *A. flavus* identified cortisone acetate, X-irradiation, and certain cytotoxic drugs, e.g., cyclophosphamide, fluorouracil, and mitomycin C, as important factors for the establishment of progressive bronchopulmonary disease (Sidransky and Friedman, 1959; Sidransky et al., 1965). Glucocorticoids, such as cortisone, triamcinolone, and hydrocortisone, alone or in combination with a cytotoxic agent, are used by numerous investigators for models of invasive aspergillosis, because the agents are well tolerated and result in dysfunction of both the polymorphonuclear cells and the monocyte-macrophage cells (Balloy et al., 2005; Sheppard et al., 2004, 2006; Steinbach et al., 2004). Cyclophosphamide is the most commonly used cytotoxic

agent for murine models to induce transient agranulocytemia, with investigators often using a combination of cytotoxic agents and glucocorticoids (Sheppard et al., 2004, 2006; Steinbach et al., 2004; Stephens-Romero et al., 2005). However, the monoclonal antibody RB6-8C5 has also been used successfully to deplete granulocytes from the circulation of mice prior to inoculation (Mehrad et al., 1999; Stephens-Romero et al., 2005). The impact of different immunosuppressive regimens, especially when evaluating the virulence of *A. fumigatus* mutants, is illustrated by the disparate results reported for mutants of *A. fumigatus* that fail to produce gliotoxin. Conflicting data from several investigators were explained when it was shown that gliotoxin null mutants have attenuated virulence only in glucocorticoid immunosuppressed mice and not in mice that are neutropenic (Bok et al., 2006; Cramer et al., 2006; Kupfahl et al., 2006; Spikes et al., 2008; Sugui et al., 2007b). This suggests that the target of gliotoxin is neutrophils via its proapoptotic activity.

Finally, the route that is used to administer the inoculum may profoundly influence the outcome of the infection. For example, mice that are injected intravenously with conidia develop disseminated disease without requiring immunosuppression (Graybill et al., 1983). The anatomical distribution of lesions following intravenous inoculation is primarily in the liver and kidney, which does not correspond with autopsy results from human cases (Bodey et al., 1992). Despite these obvious differences between the intravenous model and human disease, the ability of this model to yield reproducible and homogenous infections across entire groups of animals has resulted in its use for testing antifungal agents (Schaffner and Frick, 1985). In studies of pathogenesis, immune response, and evaluation of mutants, investigators have turned to models that more closely mimic the human disease, which is acquired by inhalation of conidia. Inhalation chambers fall into two categories, those in which the conidia are aerosolized using compressed air and those in which suspensions of conidia are aerosolized via a nebulizer. One of the advantages of using compressed air is that the surface properties of the conidia are not altered by being in suspension. However, epidemiological data supporting water and water systems as a vehicle for the nosocomial spread of aspergillosis may ameliorate the importance of that difference (Anaissie et al., 2002). A disadvantage of these models is that they may require considerable training to deliver reproducible doses of conidia (Sidransky and Friedman, 1959; Stephens-Romero et al., 2005). Inhalation chambers using suspensions of conidia range in complexity from relatively simple Plexiglas boxes to Hinner's chambers to Madison chambers (Sheppard et al., 2004, 2006; Steinbach et al., 2004). These chambers allow a large number of mice to be inoculated simultaneously, providing the homogenous groups needed for drug testing, but they require a relatively large number of conidia and multiple chambers if several strains must be compared. However, all the inhalational models share the advantage that the histological picture of discrete foci of infection distributed throughout the lungs closely mimics what is seen in human disease and the disadvantage that the exact inoculum can only be determined after the fact by quantitatively culturing the lungs of a small group of inoculated mice. Because of the need for multiple chambers to compare an isogenic set and the problem introduced if a mutant phenotype includes reduced conidiation, models in which conidia are instilled intranasally or intratracheally have been employed (Eisenstein et al., 1990; Richie et al., 2007a; Steinbach et al., 2006; Zhao et al., 2006). Although intranasal delivery results in a more patchy delivery and more bronchiolar involvement, it has still been very useful in studying mutants of *A. fumigatus*, including studies in which virulence differences are sought by assaying for direct competition between strains using mixed inocula (Aufauvre-Brown et al., 1998; Sarfati et al., 2002). The number of mouse models being employed to study invasive aspergillosis suggests that no single model is superior for all types of studies.

Other Vertebrate Models

One of the limitations of mouse models is that the small size of the animal precludes serial testing and may not allow application of imaging studies. To circumvent these obstacles, investigators have turned to rats, guinea pigs, and rabbits. As with mice, there are a number of ways to immunosuppress and inoculate these animals, although most protocols employ agents and methods modified from murine protocols. Rats have been used following cortisone immunosuppression and intratracheal instillation of conidia for numerous studies of antifungals (Schmitt et al., 1988). In order to compare the histopathology of invasive aspergillosis caused by wild-type and two mutant strains of *A. fumigatus*, cyclophosphamide-immunosuppressed rats were inoculated intratracheally with agarose beads containing the conidia, so that the origin of the lesion could be permanently marked (Shibuya et al., 1999). Guinea pigs, immunosuppressed with cortisone and cyclophosphamide and inoculated using an inhalation chamber, have been validated as an excellent model that allows for serial blood sampling, mortality testing, and tissue sampling for histopathology, quantitative PCR, and culture for organism burden (Vallor et al., 2008). Rabbits have also been used for studies of pathogenesis, drug testing, and, notably, the application of imaging modalities with implications for monitoring human disease (Petraitis et al., 2006; Spreadbury et al., 1989). Regardless of the animal model employed, it is important to make the choice that provides the best information for the purposes of the study at the cost of the fewest animals needed to produce statistically robust data.

SUMMARY

Over the past 50 years, *A. fumigatus* has emerged from obscurity into a serious problem to the medical community. A search of the PubMed/Medline database using "*Aspergillus fumigatus*" as the query reflects this dramatic change; approximately 3,000 papers can be retrieved over the past decade (1998 to 2008), which is in striking contrast to the 24 publications that are retrieved between 1950 and 1960. As we continue to discover life-saving interventions for the treatment of cancer and organ failure, the size of the immunosuppressed population is expected to increase, resulting in a greater number of individuals at risk for infection with *A. fumigatus*. Thus, novel approaches to the understanding and treatment of aspergillosis will continue to be an important need. The increased attention to this fungus is, by necessity, fueling efforts to improve the genetic tractability of the organism so that the explosion of information derived from comparative genome analyses can be used as a foundation for rigorous hypothesis testing. The challenge for the future will be to use this improved technology to unlock the many secrets contained within the *A. fumigatus* genome, particularly in the putative pathogenicity islands that have recently been identified in this species.

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Cellular and Molecular Biology of Filamentous Fungi
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Cryptococcus neoformans: Budding Yeast and Dimorphic Filamentous Fungus

JAMES W. KRONSTAD, JENNIFER K. LODGE, AND JOSEPH HEITMAN

Cryptococcus neoformans and *Cryptococcus gattii* are closely related basidiomycetous fungi that commonly infect humans to predominantly cause meningoencephalitis. Both grow as budding yeasts in the environment and in the infected host yet undergo a dimorphic transition to a filamentous monokaryon or dikaryon during sexual reproduction. Recent advances in genetic and genomic approaches have catapulted these pathogenic microbes to center stage as model systems to understand how pathogenic fungi interact with the host and their environments. Combined with recent advances in the discovery of when, where, and how sexual reproduction occurs, population genetics approaches have considerably advanced understanding of how diversity is generated and maintained. This chapter covers recent exciting advances in the field with special consideration of features of the virulence and life cycle relevant to studies of filamentous fungi and the emergence of microbial pathogens successfully infecting animals.

The immune response is not covered here because of space constraint, but there is an extensive body of literature on that topic. Excellent reviews that describe the available information on the interaction of *C. neoformans* with the host immune system have been published (Kawaki, 2004; Shoham and Levitz, 2005).

THE *C. NEOFORMANS-C. GATTII* SPECIES COMPLEX

Isolates of *C. neoformans* have traditionally been divided into the varieties *grubii* (serotype A), *neoformans* (serotype D), and *gattii* (serotypes B and C), based on biochemical and molecular markers and antigenic (serological) differences in the capsular polysaccharide (Casadevall and Perfect, 1998). *C. neoformans* and *C. gattii* are now recognized as distinct but related species as part of the *Cryptococcus* pathogenic species complex, and it is likely that additional species distinctions exist that are currently recognized as different varieties or molecular types (Kwon-Chung et al., 2002). Molecular differences between the varieties and serotypes have been examined using 28S rRNA sequence analysis, PCR fingerprinting, enzyme electrophoretic profiling, amplified fragment length polymorphisms (AFLPs), electrophoretic karyotyping, and multilocus sequence typing (MLST) (Perfect et al., 1989, 1993b; Brandt et al., 1993; Guehó et al., 1993; Wickes et al., 1994; Meyer and Mitchell, 1995; Chen et al., 1996; Meyer et al., 1999; Boekhout and van Belkum, 1997; Boekhout et al., 1997; Ellis et al., 2000; Xu et al., 2000b; Sugita et al., 2001; Fraser et al., 2005a; Bovers et al., 2008a, 2008b). For example, the PCR-fingerprinting approach with minisatellite M13- and microsatellite $(GACA)_4$- specific primers was used to group hundreds of isolates into eight major molecular genotypes (Meyer et al., 1999; Ellis et al., 2000). These are defined as follows: VNI and VNII are serotype A (var. *grubii*), VNIII is serotype AD (a hybrid serotype), VNIV is serotype D (var. *neoformans*), and VGI to VGIV are serotype B and C strains (var. *gattii*). Recent studies have also identified an additional molecular subtype for serotype A strains in Botswana, and this type is designated VNB (Litvintseva et al., 2006). In addition, more-detailed analyses have identified VGIIa and VGIIb subtypes of serotype B strains (Kidd et al., 2004). Boekhout et al. (2001) used genotyping by AFLPs to assess the genetic diversity among 207 worldwide isolates of *C. neoformans*. This work identified six major genotypic clusters, including three clusters (numbered 1 to 3) that represented *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* and three clusters (numbered 4 to 6) from *C. neoformans* var. *gattii*. Xu et al. (2000a, 2000b) performed a phylogenetic analysis of the different serotypes using MLST analysis of the mitochondrial large ribosomal subunit RNA, the internal transcribed spacer region of nuclear rRNA, and the *URA5* and *LAC1* genes. This work supported the division of strains into the three varieties or lineages (serotype A, D, and B/C) and established the evolutionary timing for their

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TABLE 1 Properties of the three varieties of *C. neoformans*

Property	Variety		
	grubii	*neoformans*	*gattii*[a]
Serotype(s)	A	D, AD	B, C
AFLP genotypes	1, 1A, 1B	2, 3	4A/B, 5A/B/C, 6A/B, 7
PCR genotypes	VNI, VNII, VNB	VNIII, VNIV	VGI, VGII, VGIII, VGIV
AIDS association	Common (predominant)	Common	Rare
Environmental niche	Soil, trees, avian feces	Soil, trees, avian feces	Soil, trees
Strain(s) with sequenced genomes	H99	JEC21/B3501A	WM276 (VGI), R265 (VGII)

[a]*C. neoformans* variety *gattii* is now recognized as the distinct species *C. gattii* (Kwon-Chung et al., 2002).

separation. That is, the *grubii* and *neoformans* varieties are separated by approximately 18.5 million years of evolution, and these varieties differ from the *gattii* variety by approximately 37 million years (Xu et al., 2000a, 2000b). On the basis of this work and phenotypic differences, *C. neoformans* var. *gattii* was reclassified as a separate species with the name *C. gattii* (Sorrell, 2001; Kwon-Chung et al., 2002). More extensive MLST analyses with larger collections of strains have recently solidified the different groupings within the *C. neoformans-C. gattii* species complex (Sugita et al., 2001; Kidd et al., 2005; Fraser et al., 2005a; Litvintseva et al., 2003, 2006, 2007; Bovers et al., 2008a, 2008b). The correlations between varieties, serotypes, and the different molecular classifications are shown in Table 1. There may be as many as six or even eight cryptic species in the pathogenic *Cryptococcus* species complex (VGI, VGII, VGIII, VGIV, VNI, and VNIV, and possibly also VNII and VNB).

THE EMERGENCE OF *C. GATTII* AS A PATHOGEN OF IMMUNOCOMPETENT HOSTS

C. gattii is the focus of considerable current interest because three molecular subtypes of this species (VGIIa, VGIIb, and VGI) have emerged as primary pathogens of humans and animals in British Columbia and the Pacific Northwest (Bartlett et al., 2007). Initially, *C. gattii* infections were noted in a variety of animals (e.g., cats, dogs, ferrets, llamas, horses, and porpoises) on Vancouver Island in British Columbia, Canada, and the first human case was documented in 1999. There have been ~250 human cases in the subsequent 10 years, and it is remarkable that the majority of these cases were in immunocompetent people. This feature of the emergence highlights a major difference between *C. neoformans* and *C. gattii* in that *C. neoformans* infections generally occur in immunocompromised individuals such as AIDS patients (Kwon-Chung and Bennett, 1984; Sorrell, 2001). Cases of *C. gattii* infection have also spread beyond Vancouver Island and are now occurring on the mainland of British Columbia and in the states of Oregon and Washington (MacDougall et al., 2007; Upton et al., 2007; Byrnes et al., 2009).

The localized appearance of *C. gattii* infections on Vancouver Island provided an opportunity for thorough and repeated environmental sampling to identify potential sources of the fungus. Systematic sampling performed in 2002 revealed heterogeneous pockets of colonization in a variety of tree species and in the soil. Repeated sampling revealed that *C. gattii* is permanently colonizing certain areas and transiently colonizing other areas (Kidd et al., 2007a). The fungus has also been found in the air and water and, rarely, in association with wild animals. That is, there does not appear to be a significant reservoir in wild-animal hosts (Duncan et al., 2006). The analysis of potential dispersal mechanisms for the fungus indicates that wood products (e.g., bark mulch), water, human movement (vehicles and shoes), and airborne spread can account for the distribution of the fungus (Kidd et al., 2007b). The isolates obtained from environmental sampling and human and animal cases have been characterized with regard to their molecular subtypes (Kidd et al., 2004, 2005, 2007a; Fraser et al. 2005a). The initial study by Kidd et al. (2004) indicated that 5% of the isolates had the VGI subtype and that 95% were VGII (of these, 90% were VGIIa and 10% were VGIIb). Sampling at other sites indicated that the proportions are variable. For example, one site yielded the VGI, VGIIa, and VGIIb subtypes in proportions of 19, 66, and 15%, respectively (Bartlett et al., 2007).

The identification of the molecular subtypes of *C. gattii* on Vancouver Island prompted more extensive studies on the global population of the species. Large collections of isolates have now been examined by PCR fingerprinting and MLST, and examples of the same genotypes found on Vancouver Island have been found in other locations. For example, the VGIIa genotype was also represented by a strain (NIH444) collected from a patient in Seattle in 1971 and from a eucalyptus tree in San Francisco in 1990 (CBS7750), and an additional isolate from California may also be related, based on four-marker MLST analysis (Kidd et al., 2005; Fraser et al., 2005a). The VGIIb subtype has been commonly identified with multiple isolates from Australia that are closely related and also one isolate from Thailand that appears to be related (based on four-marker MLST analysis) (Kidd et al., 2005; Fraser et al., 2005a). Importantly, the VGIIb isolates from Australia are part of a fertile recombining population (Campbell et al., 2005a, 2005b). The VGI genotype appears to be the most widely distributed worldwide (Meyer et al., 2003; Bartlett et al., 2007). Interestingly, the VGI isolates on Vancouver Island showed variability and an absence of a link between specific genotypes in the environmental and clinical isolates. That is, six VGI isolates from Vancouver Island were found to have four different MLST patterns and the two environmental isolates in the group were different from the clinical isolates. These results may indicate that the VGI strains causing clinical disease could potentially have been acquired during travel outside British Columbia.

The unprecedented emergence of *C. gattii* on Vancouver Island prompted speculation about the origins of the genotypes that are causing disease. As discussed by Bartlett et al.

(2007), it is possible that the fungus has long been present on Vancouver Island and that changing conditions of climate or land use have resulted in greater exposure. It is also possible that improved surveillance or the fortuitous recognition of previously undiagnosed illness has raised awareness of *C. gattii* as a primary pathogen. It has also been proposed that sexual recombination may have resulted in the formation of a hypervirulent genotype (VGIIa) that was introduced to Vancouver Island (Fraser et al., 2005a). This model is based on MLST data indicating that VGIIa and VGIIb isolates from Vancouver Island (strains R265 and R272, respectively) are identical at 14 of 30 loci examined, that the VGIIa isolates appear to be restricted to the Pacific Northwest, and that the VGIIb isolates are part of a fertile recombining population in Australia; on the fact that the vast majority of the isolates from Vancouver Island are robustly fertile in genetic crosses; on the identification of an α/α diploid isolate (RB59) that is an intermediate in same-sex mating; and on detailed analysis of the *MAT* locus of VGIIa and VGIIb documenting that the two are distinct. This model suggests that the isolates may be siblings from a mating event or that one genotype may be parental to the other. Recently, robust mitochondrial function has been linked to the virulence of the outbreak isolates (Ma et al., 2009). Whatever the origin of the strains, the emergence on Vancouver Island has made it clear that *C. gattii* is a primary pathogen with the potential to spread to new locations and to cause disease in immunocompetent humans. It is interesting that media coverage of the emergence has been relatively sparse compared with less prevalent disease agents (e.g., West Nile virus) (Nicol et al., 2008).

METABOLIC ASPECTS OF GROWTH IN THE HOST

The nutritional requirements for *C. neoformans* growth in the mammalian host have been examined in several studies. In early work, Perfect et al. (1993a) demonstrated that the *ADE2* gene encoding phosphoribosylaminoimidizole carboxylase was required for growth of the fungus in the cerebrospinal fluid of immunosuppressed rabbits. Subsequently, Rude et al. (2002) performed transcriptional profiling by differential-display reverse transcriptase PCR for *C. neoformans* cells during meningitis in an immunosuppressed rabbit model. This study revealed elevated transcripts for the gene *ICL1* encoding isocitrate lyase, a key enzyme in the glyoxylate cycle, suggesting that this cycle might be important for fungal growth in the host. However, disruption of *ICL1* did not influence growth of the fungus in the rabbit model of meningitis. Loss of the gene also did not cause a virulence defect in a murine inhalation model or cause a growth defect in macrophages (Rude et al., 2002). Similarly, deletion of the *MLS1* gene encoding malate synthase (another glyoxylate cycle enzyme) did not influence virulence in the murine model of cryptococcosis (Idnurm et al., 2007). Both *MLS1* and *ICL1* were required for growth on acetate as the sole carbon source, and these virulence assays therefore suggest that acetate utilization is not important during infection. These results are interesting because the glyoxylate cycle has been demonstrated to be important for virulence in other fungal pathogens (e.g., *Candida albicans*) and in bacterial pathogens (e.g., *Mycobacterium tuberculosis*) (Lorenz and Fink, 2001; Muñoz-Elias and McKinney, 2005). Idnurm et al. (2007) also examined whether peroxisome function was required for virulence. Two genes (*PEX1* and *PEX6*) were disrupted singly and in combination to generate mutants that were unable to grow on fatty acids but that could still grow on acetate. The *pex1* mutant showed a wild-type level of virulence in the murine inhalation model and in a wax moth virulence assay with *Galleria mellonella* larvae. Interestingly, the *pex1* and *pex6* mutants grew poorly on glucose, a result that suggested a role for hexokinase. Indeed disruption of the *HXK2* gene for hexokinase caused reduced growth on glucose. Overall, these results indicate that *C. neoformans* may be unusual in the relative importance of aspects of central carbon metabolism, such as the glyoxylate cycle, during growth in mammalian hosts.

A role for gluconeogenesis in the virulence of *C. neoformans* was uncovered by Panepinto et al. (2005) as a result of an insertional mutagenesis screen. Specifically, melanin-deficient mutants were identified upon insertional mutagenesis, and one such mutant contained an insertion in the *VAD1* gene encoding a predicted RNA-binding DExD/H-box protein. The *vad1* mutant retained residual laccase activity but was highly attenuated in a mouse model of cryptococcosis, suggesting that additional defects besides melanin production might be present in the mutant. Differential display was used as a first step to identify additional genes (besides the *LAC1* gene encoding laccase) that showed altered expression in the *vad1* mutant. One of the identified genes, *PCK1*, encoded phosphoenolpyruvate carboxykinase; disruption of this gene resulted in attenuated virulence and reduced growth on the three-carbon substrate lactate. These results suggest that lactate may be an important carbon source during infection and that gluconeogenesis is required to utilize this source during growth in the host. These conclusions are consistent with the work of Rude et al. (2002) and Idnurm et al. (2007), which indicate that utilization of two-carbon sources such as acetate is not required for growth in the host.

Additional insights into nutrient availability during infection by *C. neoformans* come from the analysis of mutants defective in functions for autophagy. This process allows cells to respond to nutrient starvation and other stress conditions by degrading proteins and organelles, and reusing the components for survival. Hu et al. (2008a) found that disruption of the *VPS34* gene encoding phosphatidyl inositol-3-kinase caused attenuated virulence in a mouse model of infection and a loss of melanin production and autophagy vesicles. Vps34 is known to be involved in vesicle transport to the vacuole and for autophagy in fungi (Kihara et al., 2001). Interestingly, loss of Vps34 resulted in rapid clearance of the mutant cells from the lungs of infected mice and rapid loss of viability upon phagocytosis by macrophages. In addition, the mutant lost viability with similar kinetics during incubation in a low-nutrient medium, suggesting that starvation sensitivity might be the reason for attenuated virulence and poor survival in macrophages. The implication is that autophagy may be a virulence-associated trait that is required for survival in the host. Additional RNA interference experiments with the autophagy-related gene *ATG8* also demonstrated a link between autophagy and virulence (Hu et al., 2008a).

In addition to requirements for carbon sources and autophagy during infection, *C. neoformans* must also obtain metals in competition with the mammalian host. Aspects of copper and iron uptake and regulation have been examined to identify the mechanisms employed by the fungus. The interest in copper uptake and homeostasis stems from the requirement for the metal in two known virulence factors, laccase for melanin production, and Cu/Zn superoxide dismutase. Waterman et al. (2007) identified a copper-dependent transcription factor, Cuf1, during a screen for

laccase-deficient mutants and went on to show that a *cuf1* disruption mutant had a growth defect on low-copper medium. The mutant did not have defects in other virulence traits (e.g., capsule formation) but did show attenuated virulence in a mouse model of cryptococcosis (by tail vein injection). Interestingly, the mutant was able to grow to levels similar to that of the wild-type strain in the lung but showed reduced growth in the brain. This finding suggests that the brain may be a particularly copper-limited environment. Consistent with this idea, Waterman et al. (2007) went on to demonstrate high expression of the gene for the high-affinity copper transporter Ctr4 in the brain. Expression of *CTR4* was also highly induced upon phagocytosis.

The competition for iron between invading pathogens and mammalian hosts is a critical aspect of infection (Schaible and Kaufmann, 2004). Pathogens have evolved high-affinity uptake mechanisms to acquire iron from iron-containing proteins (e.g., hemoglobin and transferrin), and the mammalian host employs iron sequestration as a defense strategy (Weinberg, 1999). Part of the motivation for examining iron in *C. neoformans* came from the observation that iron starvation induces capsule formation and the metal may therefore be a key regulator of virulence (Vartivarian et al., 1993). In addition, iron overload has been shown to exacerbate some infectious diseases, and this is the case for cryptococcosis (Barluzzi et al., 2002). The iron regulatory network and iron acquisition functions have been examined in some detail for *C. neoformans* (Lian et al., 2005; Jung et al., 2006, 2008; Tangen et al., 2007; Jung and Kronstad, 2008). Initially, the response of the fungus to iron deprivation was examined by transcriptional profiling (Lian et al., 2005), and this study identified general patterns of gene expression as well as specific iron-responsive functions. The latter genes encoded iron acquisition functions (e.g., an iron permease designated Ftr1 and later renamed Cft1) and a predicted mannoprotein described as a cytokine-inducing glycoprotein (Cig1) (Biondo et al., 2002). Initial analysis of the mutant defective in the iron permease indicated poor growth on low-iron medium (LIM) and delayed growth with altered capsule formation on iron-replete medium. The *CIG1* gene is interesting because the transcript was elevated 10-fold in LIM and was the most abundant message detected in cells upon iron starvation. Disruption of the gene also resulted in poor growth in LIM, and capsule formation in the mutant was no longer suppressed upon growth in iron-replete medium. Subsequent work showed that Cig1 is required for cell wall integrity (T. Lian and B. Cadieux, unpublished data).

The central transcription factor for the iron regulatory network in *C. neoformans*, Cir1, was identified based on sequence similarity to GATA-type zinc finger transcription factors that mediate iron regulation in other fungi, including Urbs1 in *Ustilago maydis* and Fep1 in *Schizosaccharomyces pombe* (Jung et al., 2006). The Urbs1 and Fep1 proteins function as negative regulators that repress the expression of iron acquisition functions under iron-replete conditions. Cir1 also appears to participate in negative regulation because disruption of the gene resulted in derepression of cell surface reductase activity. In addition, *cir1* mutants have phenotypic changes in all of the major known virulence factors of *C. neoformans*, including decreased capsule formation, poor growth at 37°C, and elevated melanin production. These results further strengthen the connection between iron and virulence. Interestingly, microarray experiments to compare the *cir1* mutant with the wild-type strain under low- and high-iron conditions revealed that Cir1 is responsible for practically all of the transcriptional response to iron. Specifically, the analysis indicated that 483 genes were downregulated and 250 were upregulated in low-iron versus high-iron media for the wild-type strain. However, no differentially expressed genes were identified in the *cir1* mutant in response to iron availability. A comparison of the transcriptional responses of the mutant and wild-type strains further indicated that 1,623 and 2,311 genes showed differential expression in high- and low-iron media, respectively. The differentially expressed genes from the microarray study were analyzed for their Gene Ontology categories, and the top groupings were for iron ion transport and siderophore transport. An examination of the genes in these categories indicated that some functions were negatively regulated by Cir1 and that other functions were positively regulated. Negatively regulated genes included some of the genes for high-affinity iron uptake (e.g., encoding iron permease and ferric reductase) and the *LAC1* and *LAC2* genes for laccase and melanin production. The positively regulated genes included siderophore transporters such as the *SIT1* gene described below. These results indicate that Cir1 may function as both a transcriptional repressor and an activator, although it is not known whether regulation is mediated in a direct or indirect fashion. Additional analysis of the microarray data linked Cir1 regulation to the cyclic AMP (cAMP), Ca^{2+}/calmodulin, and protein kinase C mitogen-activated protein kinase (PKC MAPK) signaling pathways. Other connections were revealed for sterol biosynthesis and cell wall glucan synthesis. Overall, the analysis of Cir1 placed the transcription factor in an iron regulatory network that links virulence factor elaboration to iron acquisition and a variety of cellular functions.

The characterization of gene expression in response to iron availability and Cir1 provided a list of candidate iron acquisition genes for functional analysis. These included siderophore transporter genes such as *SIT1* and two candidate iron permease genes *CFT1* and *CFT2*. It is thought that *C. neoformans* does not produce its own siderophores and that it must steal these iron-binding small molecules from other microbes. The role of the *SIT1* gene in siderophore utilization was examined by deleting the gene, and it was found that the *sit1* mutant was unable to use iron bound to the siderophore deferroxamine (Tangen et al., 2007). Interestingly, the mutant also showed altered melanin deposition and changes in the cell wall. As expected, the mutant did not show a defect in virulence in mice, indicating that siderophore utilization, at least as mediated by Sit1, is not relevant in the host. This result is consistent with the lack of siderophore production by *C. neoformans*. It is known that siderophore production is required for the virulence of the fungal pathogen *Aspergillus fumigatus* (Hissen et al., 2005; Schrettl et al., 2004). In contrast to Sit1, the iron permease Cft1 plays an important role in the virulence of *C. neoformans*. The *CFT1* and *CFT2* genes were identified by transcriptional profiling, and they both show similarity to the *FTR1* iron permease gene in *Saccharomyces cerevisiae* (Jung et al., 2008). The *CFT1* and *CFT2* genes are each paired with a putative ferroxidase gene (designated *CFO1* and *CFO2*) in the genome. In *S. cerevisiae*, the permease and ferroxidase proteins form a complex that functions in high-affinity iron uptake (Kwok et al., 2006). Initially, the expression of the *CFT1* and *CFT2* genes was examined to confirm iron dependence and regulation by Cir1 (Jung et al., 2008). Interestingly, the transcript levels for both genes are reduced at higher iron levels in culture, but the genes are regulated oppositely by Cir1. That is, the expression of *CFT1* was reduced in the *cir1* mutant and the *CFT2* transcript was higher, in agreement with the microarray studies with the *cir1* mutant.

The genes were deleted singly and together to generate mutants for functional analysis of iron source utilization and virulence. These experiments indicated that Cft1 is required for growth on inorganic iron sources and transferrin but not for growth on heme or siderophores. In contrast, Cft2 was not required for growth on any of these iron sources. Uptake assays also confirmed that Cft1 is required for uptake of radioactive iron from iron chloride and from transferring, while Cft2 again is not required. Both of the single mutants and the double mutant were tested for virulence in the murine model of cryptococcosis, and it was found that the *cft1* mutant is attenuated. Surprisingly, the loss of *CFT2* in the *cft1* mutant background further attenuated virulence, suggesting that Cft2 may play a specific role during infection. The virulence assays also revealed that the *cft1* mutant was defective in colonization of brain tissue. Overall, the analysis of Cft1 and Cft2 indicates that Cft1 is the main iron permease for iron acquisition and that transferrin or another iron source that requires Cft1 for uptake may be the main iron source in mammalian hosts (Jung et al., 2008).

GENOMES, TRANSCRIPTOMES, AND PROTEOMES

Genome Sequencing

In 1999, the National Institute of Allergy and Infectious Diseases convened a panel of experts to discuss the genomic sequencing of pathogens, and this panel agreed that the genome of *C. neoformans* was a priority for sequencing (along with *Anopheles gambiae*, *A. fumigatus*, *Cryptosporidium parvum*, *Schistosoma mansoni*, and *Staphylococcus epidermidis*). Part of the justification for *C. neoformans* was that a large, highly collaborative research community exists to make use of the sequence information. In addition, *C. neoformans* is an excellent experimental fungus with haploid and diploid phases, classical and molecular tools for genetic analysis (including DNA transformation), several selectable markers, and a gene disruption system (Heitman et al., 1999a). These features made it likely that the genome sequence would be fully exploited to understand the biology of the fungus with a focus on mechanisms of virulence.

An international genome consortium was established at a meeting in St. Louis in 1999 to develop strategies and plans for a genome-sequencing project for *C. neoformans* (Heitman et al., 1999a). Subsequent meetings of the consortium were held in St. Louis in 2001 and Vancouver in 2003, and genomics workshops became part of the International Conference on *Cryptococcus* and Cryptococcosis held in 2002 (Australia), 2005 (Boston), and 2008 (Nagasaki). The initial efforts of the consortium led to genome sequences for two related strains of the D capsular serotype, JEC21 and B3501A. The genome of JEC21 was sequenced by The Institute for Genomic Research, and the Stanford Genome Technology Center sequenced the B3501A genome. Strain JEC21 was chosen for sequencing because this strain and a congenic mating type **a** isolate (JEC20) had already been developed as genetically useful experimental strains (Heitman et al., 1999a, 1999b). Subsequently, the serotype A strain H99 of *C. neoformans* was selected for sequencing at Duke University and the Broad Institute (as part of the Fungal Genome Initiative). This strain is highly virulent in murine models of cryptococcosis and is commonly used to investigate the genetic basis of virulence. Two sequencing projects were also completed for the genomes of the *C. gattii* strains R265 and WM276. The R265 genome was sequenced by the Broad Institute, and the WM276 genome was sequenced at Canada's Michael Smith Genome Sciences Centre. This group also provided bacterial artificial chromosome (BAC) physical maps and BAC end sequences to support assembly of each of the five genomes. The *C. gattii* strains were selected because they represent the main molecular subtypes that are causing an outbreak of infections in British Columbia (the VGIIa subtype represented by R265) and that are the most common worldwide (the VGI subtype represented by WM276). Table 2 summarizes the sequencing projects for the *C. neoformans* and *C. gattii* genomes.

To date, the genomes of the serotype D strains JEC21 and B3501A have been described in a publication (Loftus et al., 2005), and manuscripts are in preparation for the other three genomes. The genome of JEC21 is approximately 20 Mb in size with 14 chromosomes ranging from ~0.8 to 2.3 Mb. These sizes are similar for the B3501A genome, except that this strain lacks an exact ~60-kb segmental duplication that is found in JEC21 (Fraser et al., 2005b). The centromeres in these genomes are thought to be regions of 40 to 100 kb that contain clusters of repetitive elements that are found only once per chromosome and contain repetitive elements unique to these regions of the genome. In fact, transposons

TABLE 2 Genome sequencing projects for *C. neoformans* and *C. gattii*[a]

Strain	Variety and serotype	Subtype (PCR)	Coverage	Sequencing center	Website for sequence access
JEC21	*neoformans*, D	VNIV	~12.5×	J. Craig Venter Institute (TIGR)	http://www.tigr.org/tdb/e2k1/cna1/
B3501A	*neoformans*, D	VNIV	~10×	Stanford Genome Technology Center	http://www-sequence.stanford.edu/group/C.neoformans/index.html
H99	*grubii*, A	VNI	~11×	Broad Institute, Duke University	www.broad.mit.edu/annotation/genome/cryptococcus_neoformans/Home.html
WM276	*gattii*, B	VGI	~6×	Canada's Michael Smith Genome Sciences Centre	http://www.bcgsc.bc.ca/cgi-bin/crypto_data/blast_wm276.pl
R265	*gattii*, B	VGIIa	~5×	Broad Institute	www.broad.mit.edu/annotation/genome/cryptococcus_neoformans_b/Home.html

[a]BAC physical maps and BAC end sequences for each strain were provided by Canada's Michael Smith Genome Sciences Centre. Expressed sequence tag sequences are also available at the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/cneo.html).

appear to represent ~5% of the genome. The parallel sequencing of both ends of 23,000 full-length cDNA clones provided an opportunity to perform accurate gene structure annotation, and this process resulted in the identification of 6,572 protein-coding genes. These contained an average of 5.3 introns and 6.3 exons per gene, thus confirming previous observations that *C. neoformans* genes are relatively intron rich. The analysis of cDNAs also revealed predicted alternative splicing for 277 genes and antisense transcripts for 53 genes. In general, the careful annotation of the JEC21 genome provides a foundation for the analysis of the other genomes of *C. neoformans* and *C. gattii* strains. In addition, the JEC21 genome provided the first microarrays for use in expression studies.

Comparative Genomics

Two studies have made use of the available genome sequences for comparative studies. In the first, Kavanaugh et al. (2006) compared the genomes of the serotype D strain JEC21 (var. *neoformans*) and the serotype A strain H99 (var. *grubii*) and discovered a 14-gene region of ~40 kb that appears to arise from a nonreciprocal transfer from *grubii* to *neoformans*. As mentioned, these varieties are thought to have diverged from a common ancestor ~18.5 million years ago, and the transfer is estimated to have occurred ~2 million years ago. The ~40-kb sequence is present in the majority of clinical and environmental isolates of var. *neoformans* from a global collection. However, two strains of var. *neoformans* that did not have the sequence were found, indicating that the introgression happened after the expansion of the variety. The proposed mechanism for the introgression of the sequence is that an incomplete sexual liaison occurred between strains of the two varieties and that the exchange occurred in a hybrid intermediate. Repetitive elements are associated with the transferred region, and these sequences, in particular the non-long-terminal-repeat retrotransposable element Cnl1, may have participated in the introgression. Overall, this work indicates that rare introgression events may contribute to genetic exchange and speciation in *C. neoformans*.

A second study employed the genomes of strains JEC21 and H99 and comparative genome hybridization (CGH) to examine the extent of variation in isolates of different mating type, different ploidy, and different molecular subtype within varieties (Hu et al., 2008b). Tiling arrays were developed using NimbleGen, Inc. technology such that unique oligonucleotides of 45 to 85 bp in length were spaced at an average interval of 44 bp on one strand of each chromosome. Initially, the arrays were employed to compare the relationship between the log2 ratio of hybridization on the arrays and the percent sequence identity for genes in the ~100-kb *MAT***a** and *MAT*α mating-type loci. This calibration provided a means to distinguish regions of sequence divergence from deletions. Subsequently, the tiling array for the JEC21 genome was used to examine two serotype D genomes from the strains NIH12 and NIH433. These strains were the progenitors of a cross that was used to initiate a series of backcrosses that eventually yielded JEC21 (Heitman et al., 1999b). Therefore, approximately 50% of the genetic content of JEC21 should derive from each of the progenitor genomes, and the CGH data revealed that this was indeed the case. This result indicated that CGH was sufficiently sensitive to detect sequence divergence (on the order of 2%) between strains of the same variety and serotype.

The tiling array for the genome of the serotype A strain H99 (var. *grubii*) was employed to compare the genomes of different molecular subtypes within one serotype or variety. As mentioned above, isolates of var. *grubii* have been divided into three molecular subtypes by PCR fingerprinting, AFLP analysis, and MLST studies. The genomes of representatives of these VNI, VNII, and VNB types were compared with the genome of H99, a VNI strain, and extensive variation was observed, such that VNII and VNB strains were more variable than VNI strains. This result matches the expectations of the original classification of the strains. The variation takes the form of regions of difference that include segments of divergent sequence, deletions, and amplifications. In addition, it was found that the VNI strain CBS7779 and the VNII strain WM626 are partially disomic for chromosome 13 (chr 13). This result was confirmed for WM626 by integrating a marker on chr 13 and characterizing the genotypes of the transformants and by quantitative reverse transcriptase PCR for both strains. Chromosome copy number variation has not been previously reported for *C. neoformans*, although karyotype changes have been well documented (Fries et al., 1996). Both CBS7779 and WM626 are clinical isolates, and the discovery of disomy suggests that this type of variation may occur in infected patients.

The tiling arrays for the JEC21 and H99 genomes were both used to characterize three strains with the hybrid AD capsular serotype (strains CDC228, CDC304, and KW5). As noted previously, these hybrids are diploid or aneuploid (Lengeler et al., 2001) and they arise via mating between serotype A and D strains. It was of interest to further define their chromosome content by using CGH, and the results indicated that the hybrid strains did not retain a full set of chromosomes from each parent. Specifically, all three strains only had the serotype A version of chr 1, suggesting that the serotype D version had been lost. In addition, chrs 6 and 7 of KW5 appeared to be the serotype A version, and chr 8 was from the serotype D genome. For strain CDC304, the serotype D version of chr 5 was retained. These results were confirmed for the three strains by PCR-restriction fragment length polymorphism analysis, and this approach was used to extend the analysis to 16 additional strains. It was found that 11 of the strains preferentially retained the serotype A version of chr 1 and the other 5 strains had both the serotype A and D versions. These results indicate that there may be a selective advantage to retaining only the serotype A version of this chromosome.

Transcriptome Analysis

The availability of the genome sequences and molecular techniques for *C. neoformans* strains provides an opportunity to define the transcriptome of the pathogen under a variety of growth conditions. Studies to examine differential expression beyond the level of single genes have employed subtractive methods, differential display, serial analysis of gene expression (SAGE), and microarrays. A subtraction PCR method was used by Cox et al. (2003) to identify the transcript for a Cu,Zn superoxide dismutase that was expressed preferentially at 37°C. The corresponding gene, *SOD1*, was required for the fungus to withstand reactive oxygen species, for full virulence in a mouse model of disease, and for wild-type levels of growth in macrophages. The differential-display technique has been used to identify genes with elevated transcripts during the growth of *C. neoformans* in the central nervous system of rabbits with experimental meningitis (Rude et al., 2002). As mentioned earlier, this work resulted in the identification of the *ICL1* gene encoding isocitrate lyase. It also identified the gene *APP1*, which is regulated by the *IPC1* gene encoding phosphoryl ceramide synthase 1, an enzyme in the sphingolipid pathway (Luberto et al., 2003). The *IPC1* gene is known to influence melanin formation and growth of the fungus in macrophages, while loss of *APP1*

results in altered virulence in mouse models. As mentioned earlier, differential display was used to identify genes with altered expression in the *vad1* mutant; this approach revealed elevated transcripts for the transcriptional regulator Not1 and reduced transcription for the *PCK1* gene encoding phosphoenolpyruvate carboxykinase, the *TUF1* gene for mitochondrial protein synthesis, and the *MPF3* encoding a function involved in cell wall integrity (Panepinto et al., 2005). Other applications of differential display include the identification of the gene encoding the MAT**a** pheromone (McClelland et al., 2002) and the description of 29 genes whose expression changed in response to the antifungal pentapeptide auristatin PHE (Woyke et al., 2004).

A series of SAGE experiments were performed to develop a view of transcriptional changes relevant to virulence. The SAGE procedure involves preparing libraries of short sequence tags (e.g., 14 bases) that represent the mRNAs present in cells grown under different conditions (Velculescu et al., 1995). The frequency of each tag provides an indication of the abundance of the corresponding transcript. Initially, libraries were prepared and sequenced for strains JEC21 and H99 grown at 25 and 37°C (Steen et al., 2002). The results from these libraries suggested that temperature influenced the expression of histone genes, perhaps indicating a change in chromatin packaging, and that the response to growth at 37°C includes elevated expression of heat shock proteins, translation machinery components, mitochrondrial proteins, and stress proteins, such as superoxide dismutase. A follow-up SAGE study was performed with *C. neoformans* cells (strain H99) collected directly from the cerebrospinal fluid of infected rabbits (Steen et al., 2003). This analysis revealed that the fungus was actively engaged in protein synthesis, protein degradation, small-molecule transport, and signaling during infection. In addition, a large number of tags matched putative genes for energy production, suggesting active growth in the host. As described earlier, SAGE was also used to examine the response of *C. neoformans* to iron deprivation (Lian et al., 2005; Hu et al., 2007). This analysis was performed for the serotype D strain B3501A, and later work employed the low-iron growth condition for the serotype A strain H99, as well as mutants defective in the regulatory and catalytic subunits of protein kinase A (PKA) (Hu et al., 2007). Comparison of the wild-type cells with the two mutants revealed that PKA influences transcript abundance for genes involved in cell wall synthesis, transport functions (including iron uptake), the tricarboxylic acid cycle, and glycolysis. Differential expression of ribosomal protein genes, stress and chaperone functions, secretory pathway components, and phospholipid synthesis was also observed. These changes prompted follow-up experiments that demonstrated differential responses of the PKA mutants to temperature, lithium choride, and caffeine. Overall, the SAGE data collected from all of the conditions provide a large set of differentially expressed genes for subsequent analysis (e.g., as targets for transcription factor discovery), and the data also support genome annotation efforts by identifying transcription units.

Several studies have employed custom or community-constructed microarrays for the analysis of differential transcription in *C. neoformans*. For example, Kraus et al. (2004) employed a custom array of 130 PCR products of cDNAs from JEC21 and H99, along with 6,144 genomic clones of H99, to study the response of *C. neoformans* to growth at 37°C. This work identified a transcription factor, Mga2, which was induced at 37°C, as well candidate targets encoding fatty acid biosynthetic enzymes. The same microarray was employed by Fan et al. (2005) to examine transcriptional changes at 2 and 24 h after phagocytosis of fungal cells (strain H99) by the J774A.1 macrophage-like cell line. This analysis revealed upregulation of transcripts for several transporters (for sugars, amino acids, and iron), oxidative stress response functions, and autophagy and lipid metabolism. Many of the genes in the mating-type locus also showed elevated expression at 24 h. Conversely, genes for ribosomal functions, rRNA processing, and translation had reduced expression. Further experiments with the genomic array examined changes in gene expression in response to mutation of the *GPA1* gene, encoding a Gα subunit upstream of the cAMP pathway in *C. neoformans* (Pukkila-Worley et al., 2005). This work identified a link between Gpa1 and several genes for capsule synthesis and made the discovery that two genes, *LAC1* and *LAC2*, encode laccases regulated by the cAMP pathway (see also Missall et al., 2005). These results are consistent with functional studies indicating that the cAMP pathway regulated both capsule and melanin formation (D'Souza et al., 2001). Another type of custom array designed with 70mer oligonucleotides for gene models from the H99 genome was employed by Chow et al. (2007) to explore the transcriptional response to heat shock, growth at 37°C, and nitric oxide (NO) stress. This study identified heat shock and other stress-related proteins that responded to temperature, as well as a *Cryptococcus*-specific set of four genes that were regulated by NO stress. Additional genes that responded to NO included the previously characterized Fhb1 protein (de Jesús-Berríos et al., 2003), catalase, oxidoreductases, and mannitol-1-phosphate dehydrogenase.

As described earlier, microarrays have been used to examine the response of *C. neoformans* to growth in media with different iron levels and to a defect in the iron regulator Cir1 (Jung et al., 2006). This work employed the community array designed with the JEC21 genome that is available from the Washington University Genome Sequencing Center. This array was also used by Missall et al. (2006) to investigate the response of strain H99 to a 2-h exposure to acidified sodium nitrite. The comparison of cells with and without exposure revealed changes twofold or greater in the transcript levels of 421 genes (205 were downregulated and 216 were upregulated). These genes encoded functions for cell wall organization, stress response, metabolism, transport, amino acid biosynthesis, and respiration. The JEC21 microarrays were also employed by Chang et al. (2007) to investigate the role of the *SRE1* gene of *C. neoformans* in the response to low oxygen. *SRE1* encodes a homolog of the mammalian sterol regulatory element binding protein, and low oxygen activates the Sre1 protein. Low-oxygen conditions also decrease sterol synthesis, and the microarray analysis confirmed that Sre1 activates genes for ergosterol biosynthesis as well as iron uptake. Functional studies revealed that the *sre1* mutant is sensitive to azole drugs that target the ergosterol pathway and that the *SRE1* gene is required for virulence. In general, use of microarrays for *C. neoformans* is just beginning, but the results to date illustrate the power of integrating genome-wide studies with subsequent molecular genetic approaches to test the functions of differentially expressed genes.

Proteomic Studies with *C. neoformans*

Several investigations have made use of the genomes to interpret proteomic data. The study of the response to nitrosative stress described above included proteomics analysis by two-dimensional gel electrophoresis and mass spectrometry in parallel with the microarray analysis

(Missall et al., 2006). In the protein expression experiment, treatment with sodium nitrite resulted in changes in the levels of 32 proteins, of which 27 were identified by peptide mass fingerprinting. These proteins included thioredoxin and the glutathione antioxidant system, stress-related oxidoreductases, and proteins involved in amino acid biosynthesis, transcription, translation, metabolism, and signaling. A proteomics approach was also used to explore the glycosylphosphatidylinositol (GPI)-anchored mannoproteins and proteases that were cell-bound or extracellular for *C. neoformans* (Eigenheer et al., 2007). This study made use of an acapsular mutant and employed trypsin and β-glucanase to remove proteins for analysis by liquid chromatography-mass spectrometry. A total of 29 extracellular proteins were identified that had signal sequences, and 17 of these had a predicted GPI anchor motif. Many (14) of the 17 proteins were also predicted to be GPI anchored by Levitz and Specht (2006). Several of the proteins identified by the proteomics approach were proteases, and these included serine, aspartic, and metalloproteases. The combination of the proteomics analysis and previous studies (Eigenheer et al., 2007; Biondo et al., 2006; Levitz and Specht, 2006) suggested that at least one-half of the extracellular proteins of *C. neoformans* are mannoproteins.

An interesting recent study on extracellular proteome targeted proteins associated with extracellular vesicles (Rodrigues et al., 2007, 2008). These extracellular vesicles have been termed "virulence bags" because they contain the capsule polysaccharide that may be used for capsule growth as well as proteins that contribute to virulence (laccase and urease). The proteomic analysis of the vesicles identified 76 proteins for functions that included chaperone activity, signal transduction, ribosome biogenesis, sugar and lipid metabolism, protein/amino acid metabolism, and formation of the cytoskeleton. Several proteins with miscellaneous functions were also identified. Rodrigues et al. (2008) suggest that the vesicles arise from fungal exosomes and that they may function not only to deliver material to the extracellular space for the elaboration of virulence factors (e.g., capsule and melanin) but also to deliver material into host cells during infection.

THE SEXUAL CYCLE OF *CRYPTOCOCCUS* INVOLVES MORPHOGENIC AND DIMORPHIC TRANSITIONS BETWEEN YEAST AND HYPHAL GROWTH MODES

Among the pathogenic fungi, *C. neoformans* is a common opportunistic pathogen of immunocompromised hosts that can also infect seemingly healthy hosts (Casadevall and Perfect, 1998). The most commonly observed growth mode is as a budding yeast, both in the environment and in infected animals and patients. Yet the organism can also undergo a dimorphic transition during the sexual cycle, adopting a dikaryotic hyphal growth mode with striking similarities to key aspects of the growth of filamentous fungi (Hull and Heitman, 2002). In addition, cellular responses to pheromone during the early stages of mating lead to the formation of elongated conjugation tubes (Davidson et al., 2000), and atypical diploid isolates are thermally dimorphic, growing as a yeast at 37°C and as a hyphal filamentous fungus at lower growth temperatures (Sia et al., 2000). Thus, a variety of key cell biological questions of import remain to be addressed, involving cellular morphogenesis and polarity, how nuclear migration occurs during mating and hyphal growth, the formation and role of clamp cells in nuclear partitioning of the dikaryon, and in the mechanisms and signals that trigger formation of the terminal fruiting bodies, the basidia, in which nuclear fusion and meiosis occur, ultimately resulting in the formation of four long chains of spores representing the suspected infectious propagules.

This pathogenic microbe is ubiquitous; virtually all individuals have been exposed by inhalation of spores or desiccated yeast cells, and some harbor a dormant latent granulomatous form in the hilar lymph nodes (Casadevall and Perfect, 1998). Reactivation occurs in response to immunosuppression from AIDS, steroids, organ transplantation, or malignancy. The organism disseminates to infect most prominently the central nervous system. *Cryptococcus* has a laboratory-defined sexual cycle (Kwon-Chung, 1975) but has never been directly observed mating in nature. The vast majority of isolates are one (α) of two mating types, and thus, until recently it has been unclear what role sex plays in this pathogenic microbe. The discovery of unisexual reproduction in the laboratory (Lin et al., 2005) provides insights into how diversity might be generated and maintained within a population that consists of almost exclusively a single mating type, and this may be the route by which infectious spores are produced in nature. We now appreciate that mating occurs in nature in environments in which both opposite mating types are present, and also in other environments from which only the α mating type has been isolated.

C. neoformans is most commonly isolated as a haploid yeast and has a laboratory-defined bipolar mating system with two mating types, **a** and α (Hull and Heitman, 2002; McClelland et al., 2004; Wickes, 2002) (Fig. 1). Nutrient limitation, or the presence of inositol, stimulates production of pheromones that trigger the formation of long projections known as conjugation tubes (Xue et al., 2007). In some cases, both mating partners produce conjugation tubes, whereas in other situations the α mating partner produces a conjugation tube and the **a** partner becomes enlarged (Fig. 2). In both cases, these morphological events result in cell-cell fusion, potentially in an asymmetric fashion. Nuclear migration and congression occur, but karyogamy is delayed under most conditions. The resulting dikaryon then undergoes a dimorphic filamentous transition to produce abundant hyphae in which each compartment contains two nuclei, one from each parent. Coordinated nuclear migration and pairing involve the formation of a special cell type, known as the clamp cell, which links adjacent cells in the hyphal filament (Fig. 3). Clamp cells form as a protrusion from the hyphal tip cell, much like a bud in the yeast form. One of the two nuclei migrates into the clamp cell, both nuclei divide, septa are then formed, and the clamp cell tip bends back to the filament and fuses with the penultimate filament cell formed by septation. Clamp cell fusion is thought to involve localized pheromone production and sensing, and thus, the sexual cycle involves both an initial cell-cell fusion event and then repeated rounds of pheromone-dependent clamp cell-hyphal cell fusion at each division. This process ensures that each hyphal compartment receives two nuclei, one from each parent, enforcing the fidelity of nuclear migration. Why the organism goes to so much trouble to maintain the dikaryon is less clear. Hypotheses that have been advanced are that the dikaryon has a growth or selective advantage; that branching of the hyphae can occur at subterminal cells, necessitating that the dikaryon be stably maintained; or that this increases the options for introgressing nuclei from other fusion events to establish optimal dikaryotic nuclear relationships. In any case, the genetic pathways that govern the formation and fusion of clamp cells, which are directly analogous to the

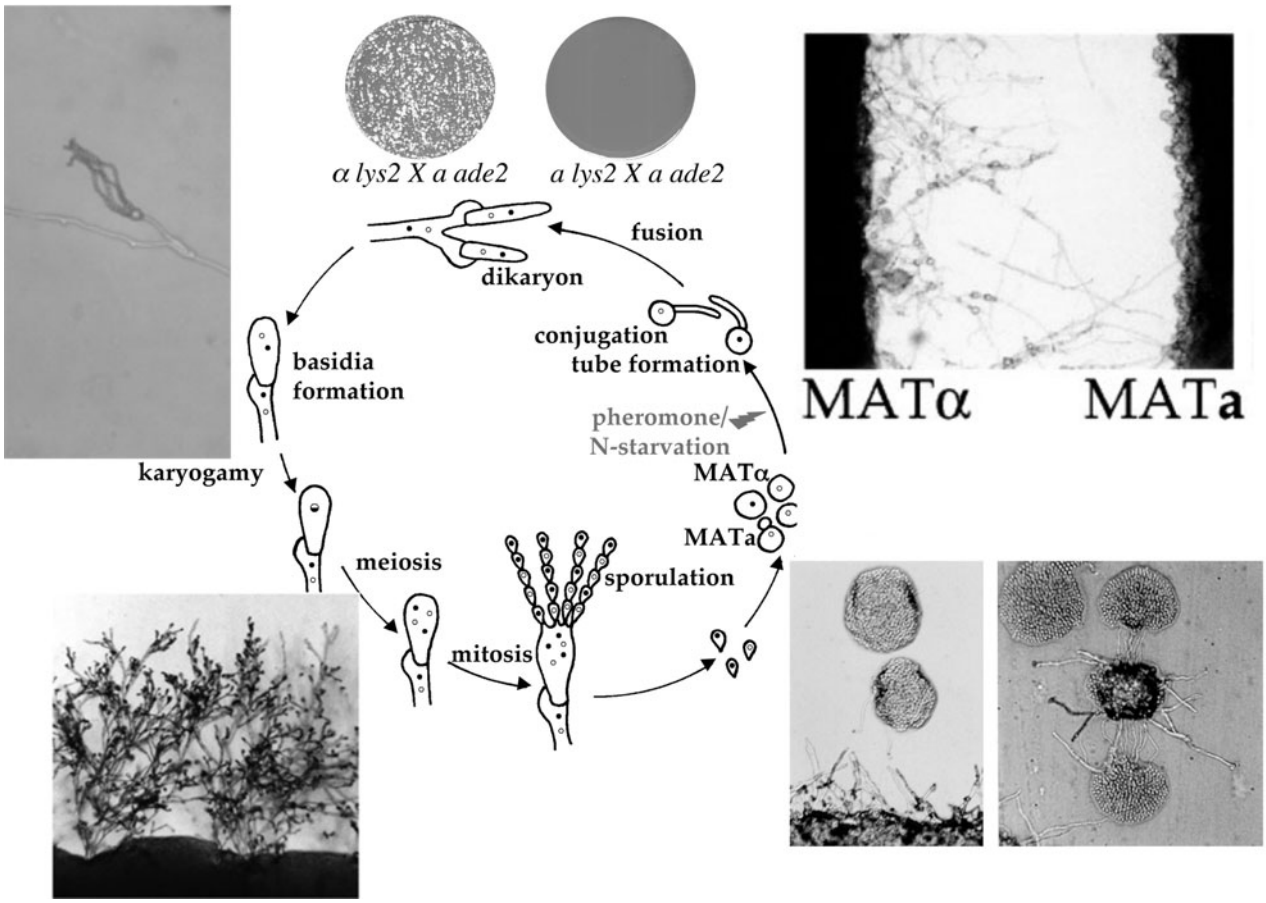

FIGURE 1 The morphology of sexual reproduction in *C. neoformans*. A diagram representing the stages in sexual reproduction for *C. neoformans* (center) is surrounded by images showing representative stages in the process. Two haploid cells of opposite mating types (α and **a**) respond to a panoply of appropriate environmental cues (including nutrient limitation, the presence of inositol and copper ions, desiccation, darkness, low temperature, low levels of carbon dioxide, surface growth, the presence of plants, or growth on pigeon guano) that stimulate pheromone production and early morphological changes including conjugation tube formation that lead to cell-cell fusion. Following cell-cell fusion, the nuclei congress, but nuclear fusion is delayed, and the resulting dikaryon switches from growth as a budding yeast to growth as a dikaryotic hyphae. Unknown signals trigger the production of terminal fruiting structures, the basidia, wherein karyogamy and meiosis occur, and long chains of infectious basidiospores are then produced by basipetal budding from the basidium. Germination of spores produces haploid meiotic products that return to the budding yeast growth mode. Images show (counterclockwise from the lower right hand corner) (i) microcolonies producing conjugation tubes oriented towards dikaryotic hyphae from a mating mixture as a source of pheromones (left panel) and microcolonies on the surface of V8 mating medium linked by conjugation tubes; larger dikaryotic filaments are also seen emanating from the central microcolony of **a** cells as a result of cell-cell fusion mediated by conjugation tubes; (ii) confrontation assay on filament agar medium showing the formation of conjugation tubes and monokaryotic fruiting by the α partner and the production of enlarged cells by the **a** partner; (iii) fusion assay, in which the production of prototrophic dikaryons or diploids following cell-cell fusion is monitored by growth on minimal medium lacking lysine and adenine; (iv) dikaryotic filament with a terminal basidium decorated with four long intertwined spore chains; (v) edge of a mating mixture on V8 mating medium showing profuse dikaryotic hyphae with terminal basidia and spore chains (not resolved at this magnification).

croziers of euascomycetes, remain a fertile area for future research.

Another interesting feature of sexual reproduction in *Cryptococcus* is that mitochondria are inherited in a uniparental fashion from the parent of **a** mating type (Fig. 4) (Xu et al., 2000a, 2000b; Yan and Xu, 2003). While it is often the case that mitochondria are similarly uniparentally inherited in anisogametic species, such as humans, in which it is the oocyte rather than the sperm that contributes mitochondria to the embryo, in isogametic species in which the two gametes are morphologically identical, such as *S. cerevisiae*, there is often biparental inheritance of mitochondria. How uniparental mitochondrial inheritance occurs is not well understood. Given the anisometry in the initial fusion events during mating of *Cryptococcus*, this may involve preferential movement of nuclei but not the mitochondria from

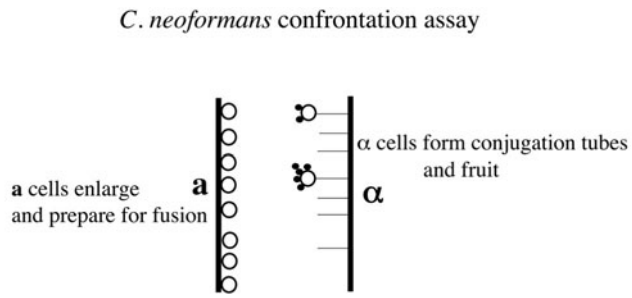

FIGURE 2 *C. neoformans* mating partners respond to mating pheromones during confrontational growth. The diagram depicts morphological events transpiring during the early events in sexual reproduction that can be detected using a confrontation assay in which mating partners are grown in close proximity but not touching on V8, SLAD (super low ammonium dextrose), or filamentation agar. With serotype D strains, α cells respond to **a** pheromone to form conjugation tubes and then undergo monokaryotic fruiting, which may serve as a response to locate more-distant mating partners. By contrast, **a** cells often undergo an isotropic expansion in response to α pheromone to form enlarged cells, possibly to serve as targets for fusion by conjugation tubes or hyphae produced by the α mating partner. Under other conditions, or with other isolates, **a** cells can also be observed producing conjugation tubes in response to α pheromone.

the α parent into the **a** parent (the benign-neglect model). Alternatively, this might involve an active destruction of one mitochondrial genome or a preferential protection of the mitochondrial genotype that survives. Recent molecular studies have revealed that both *MAT* locus-encoded homeodomain proteins, Sxi1α and Sxi2**a**, are required (Yan et al., 2004, 2007), but their downstream targets in a pathway controlling mitochondrial inheritance remain to be elucidated. Moreover, the role of uniparental mitochondrial inheritance is also unclear but may reflect a mechanism to protect the organism from invasion from inteins within the mitochondrial genome.

Nuclear fusion and meiosis ultimately occur in specialized structures, the basidia, and chains of potentially infectious spores bud from the basidium. The signals that trigger basidium formation are not known, nor are the specific proteins and gene products involved. Four long chains of spores are produced, and micromanipulation of spores from individual basidia and dissection down spore chains reveal that adjacent spores are often genetically distinct, indicating that the four postmeiotic nuclei in the basidia are mobile and can contribute their daughter nuclei to any of the four different spore chains (Kwon-Chung, 1980). Spores are readily aerosolized, deposit in alveoli of the lung, and can be up to 100 times more infectious than yeasts (Sukroongreung et al., 1998). Recent studies document that spores are infectious (Botts et al., 2009; Giles et al., 2009; Velagapudi et al., 2009). Whether spores are infectious propagules in nature is

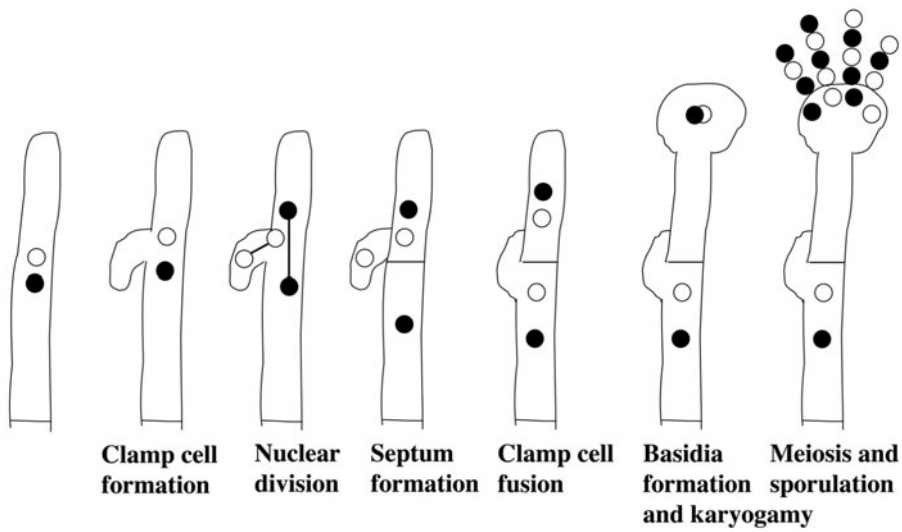

FIGURE 3 Clamp cells ensure faithful nuclear segregation during dikaryotic hyphal growth. During hyphal growth of the dikaryon, a specialized cell known as a clamp cell plays a central role in ensuring that each hyphal cell receives one copy of each nucleus. Stages in clamp cell formation, nuclear migration and division, and clamp cell fusion are depicted. Based on studies in other basidiomycetous fungi, it is hypothesized that pheromone production and sensing are involved in the clamp cell-hyphal cell fusion events. Nuclear fusion (karyogamy) is depicted occurring in the terminal basidium, followed by meiosis and mitotic production of long chains of basidiospores. The two opposite-mating-type nuclei are depicted with filled and solid circles.

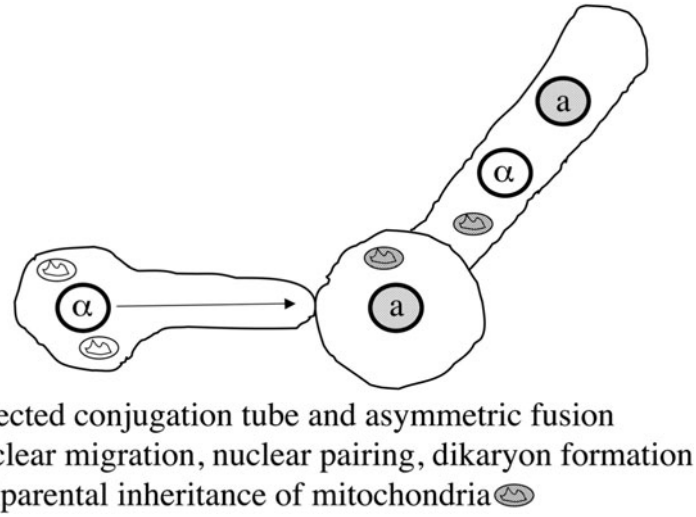

FIGURE 4 Mitochondria are inherited in a uniparental fashion during sexual reproduction. Directed growth of the conjugation tube from an α mating partner towards the recipient **a** mating partner is depicted. Mitochondria are depicted with oval symbols; shaded symbols indicate those from the **a** parent, and open symbols depict those from the α parent. Analysis of mitochondrial genotypes from meiotic progeny produced by sexual reproduction reveals uniparental inheritance from the **a** parent. Mitochondria from the α parent either may be left behind if only the nucleus migrates through the conjugation tube or may be actively destroyed, or both. Recombination between mitochondrial genomes has also been observed in some defined genetic crosses.

unknown. Recent studies provide evidence that sexual reproduction occurs in nature and may involve interactions with common environmental niches, including both pigeon guano and plants (Nielsen et al., 2007; Xue et al., 2007).

THE MATING-TYPE *(MAT)* LOCUS AS A PARADIGM FOR GENE CLUSTER EVOLUTION

The **a** and α alleles of the *Cryptococcus* mating-type locus (*MAT*) have been cloned and sequenced, revealing several unusual features (Fraser et al., 2004, 2005a; Karos et al., 2000; Lengeler et al., 2002; Ren et al., 2005). The locus spans more than 100 kb and encompasses some 25 genes, most as divergent alleles in the opposite mating types. The only *MAT* genes that are unique to each mating type encode the homeodomain proteins Sxi1α and Sxi2**a**, which are required for completion of the sexual cycle and which define the **a**/α cell type (Hull et al., 2002, 2005). The locus has undergone multiple rearrangements and is punctuated by five essential genes that constrain evolution by ensuring that deletions involving these essential genes would be counterselected (Fraser et al., 2004).

The sequence and comparative analysis of the serotype B *C. gattii* mating-type locus alleles, including Australian VGI environmental isolates (WM276 and E566) and a Vancouver Island outbreak VGII clinical isolate (R265) (Fraser et al., 2004, 2005a), provided insights into evolutionary events that contributed to fashion this unusual genomic region. This comparative genomic analysis supports an evolutionary model involving an ancestral tetrapolar mating system (two unlinked *MAT* loci) into which genes of related function were acquired, followed by assembly into a contiguous gene set by mechanisms hypothesized to have involved chromosomal translocation, recombination, and ongoing gene conversion events (Fraser et al., 2004; Hsueh et al., 2006). Recombinational activators that flank the *MAT* locus and also lie within it were discovered, and this provides a molecular foundation to understand at a molecular level how gene clusters evolve and function (Hsueh et al., 2006). Direct experimental support for the hypothesized tetrapolar ancestor and for the tripolar intermediate has been provided by a series of studies in which the *SXI1*α and *SXI2***a** genes were deleted from *MAT* and relocated to an unlinked genomic locale (Hsueh et al., 2008). This analysis reveals that *Cryptococcus* can complete its sexual cycle as a tetrapolar fungus and also provides evidence that the tripolar intermediate was likely to have been an unfavored transient state, based on the production of a higher proportion of sterile progeny from defined genetic crosses. The understanding derived from studies of the *Cryptococcus MAT* locus extends also to serve as a paradigm for evolution and function of fungal gene clusters involved in metabolism and secondary metabolite production (Bok et al., 2006; Gardiner et al., 2004; Keller et al., 2005; Wong and Wolfe, 2005), and the processes by which sex-determining regions and even sex chromosomes evolved via similar molecular events in organisms as diverse as fungi, insects, plants, and animals (Fraser and Heitman, 2005).

Mating type of *Cryptococcus* has been associated with virulence, and α cells are more pathogenic in a murine model than congenic **a** cells in the serotype D var. *neoformans* lineage (Kwon-Chung et al., 1992; Nielsen et al., 2005b). Strains of α mating type predominate in environmental and clinical isolates (Kwon-Chung and Bennett, 1978), but laboratory crosses produce an equal proportion of the two mating types (Kwon-Chung, 1976a). The contribution of the α allele to pathogenicity is background dependent, and virulence is a quantitative trait, in which *MAT* interacts with other unlinked genes to contribute to virulence (Nielsen et al., 2005b). Several *MAT* locus gene products have been linked to virulence potential of *Cryptococcus*, including the transcription factor Ste12, the PAK kinase Ste20, and the Ste3 pheromone receptor, but allele differences that contribute to virulence differences between α and **a** isolates remain to be determined (Chang et al., 2000, 2003; Wang et al., 2002; Yue et al., 1999). In the most common pathogenic variety, serotype A, there is no intrinsic difference in virulence of

cells of opposite mating types when they are infected individually for the KN99α/KN99**a** strain pair congenic with H99, but during coinfection α cells have an advantage in crossing the blood-brain barrier (Nielsen et al., 2003, 2005a). *MAT*-encoded genes are induced following phagocytosis by macrophages and in the central nervous system, and pheromone signaling is hypothesized to enable a dialog between cell types during coinfection that influences virulence by a process analogous to quorum sensing in bacteria (Del Poeta et al., 1999; Fan et al., 2005; Nielsen et al., 2005a) and pathogenic fungi, most notably *C. albicans* (Chen et al., 2004; Hornby et al., 2001).

SEX AND THE GENERATION OF INFECTIOUS PROPAGULES

In addition to virulence differences in the host, the mating-type locus governs completion of the sexual cycle, which may contribute to virulence by enabling production of infectious spores. Worldwide, *C. neoformans* is ubiquitously associated with pigeon guano. The majority of the world's population has been exposed via inhalation. The infectious propagule is desiccated yeast cells or spores; both are small enough to reach alveoli and are not subject to efficient mucociliary clearing. Particles small enough to be spores are present in areas contaminated with dried pigeon guano (Neilson et al., 1977; Ruiz and Bulmer, 1981) and in the air on Vancouver Island, where *C. gattii* is now endemic (Kidd et al., 2004). As few as 50 spores are infectious and lethal in a murine model (Zimmer et al., 1984) and when aerosolized were reported to be up to 100-fold more infectious than yeast cells (Sukroongreung et al., 1998), although recent studies reveal virulence similar to yeast cells (Botts et al., 2009; Giles et al., 2009; Valagapudi et al., 2009). Thus, spores represent efficient infectious propagules.

But if spores are the infectious agent, how they are produced in nature remains a central question. Spores are efficiently produced when **a** and α cells mate. Mating occurs on pigeon guano medium and in association with plants and woody debris (Botes et al., 2008; Nielsen et al., 2007; Ren et al., 2006; Xue et al., 2007), indicating that infectious spores could be produced via opposite-sex mating in environmental niches. However, most isolates are α and an asexual life cycle could have been selected concomitant with emergence as a successful pathogen. One argument is that virulence is a polygenic trait, and sexual reproduction would therefore be disadvantageous. However, the first isolate of the unusual **a** mating type in the predominant pathogenic form (serotype A var. *grubii*) was discovered as a clinical isolate from Tanzania (Lengeler et al., 2000). Subsequently, an unusual population was found in Botswana, in which up to 25% are **a** mating type, the population exhibits genetic signatures of recombination, and these isolates are robustly fertile in the lab (Litvintseva et al., 2003). Thus, the sexual cycle has not been lost but rather geographically restricted to sub-Saharan Africa. There, the large immunocompromised AIDS population may be exposed to meiotic recombinants, which may differ in virulence potential.

But even if the sexual cycle remains extant in sub-Saharan Africa, the vast majority of isolates worldwide are α, and **a** isolates are rare or absent. Thus, most isolates are precluded from participating in the traditional **a**-α sexual cycle. Monokaryotic fruiting is an alternative differentiation cascade sharing many features with sexual reproduction but occurs when α cells are cultured alone on mating medium (Erke, 1976; Wickes et al., 1996). This finding provided a simplifying hypothesis: α strains undergo monokaryotic fruiting to produce infectious spores. This remains an attractive model; however, many strains do not undergo fruiting, and it was recently reported that fruiting is not limited to α strains, raising questions about the relevance of this pathway in the infectious cycle (Tscharke et al., 2003). The discovery that monokaryotic fruiting represents a novel form of the sexual cycle involving cells of only one mating type (Lin et al., 2005) and the finding that the α allele of the *MAT* locus represents one of several quantitative trait loci promoting fruiting (Lin et al., 2006) stimulated reinvestigation of the roles this pathway plays in evolution and virulence and of specific functions of the mating-type locus in biology and virulence of this pathogenic microbe.

Monokaryotic fruiting is a novel sexual cycle involving diploidization, filamentous growth, and genome reduction to haploid basidiospores by a process involving recombination and conserved meiotic components (Dmc1 and Spo11) (Lin et al., 2005) (Fig. 5). Nuclear fusion can occur early in the process, generating diploid intermediates; in other strain backgrounds or conditions, nuclear fusion appears to occur later, possibly only in the basidium, analogous to opposite-sex mating. Although it was originally thought that monokaryotic fruiting was strictly haploid and asexual (Wickes et al., 1996), it remains an open question whether asexual sporulation occurs in *Cryptococcus*, given recent evidence that the process can involve whole-genome ploidy changes (haploid-diploid-haploid), that frequent recombination is at a level on par with meiosis during **a**-α crosses, and, finally, that key meiotic components are required for efficient sporulation and spore viability during monokaryotic fruiting. It will be of considerable interest to explore whether some type of asexual sporulation also occurs, analogous to asexual conidiation in *A. nidulans*, *N. crassa*, and other filamentous euascomycetes.

C. neoformans is thus functionally both heterothallic (self-sterile and outbreeding) and homothallic (self-fertile and inbreeding). The ability of α cells to engage in sex without an **a** partner could have driven the disparity between the two mating types in nature or have arisen as a consequence thereof. Both heterothallic and homothallic life cycles coexist in other fungi, such as the model yeast *S. cerevisiae*, in which Ho^+ strains are homothallic, whereas naturally occurring ho^- mutants are heterothallic (Mortimer, 1993a, 1993b). Moreover, the requirement for heterozygosity at the *MAT* locus for meiosis and sporulation in *S. cerevisiae* can be bypassed by mutations in the gene encoding the Rme1 repressor of meiosis (Covitz et al., 1991; Mitchell and Herskowitz, 1986). These findings invite further investigation of the roles that the mating-type locus plays in the life cycle, sexual cycle, and virulence cycles for *C. neoformans*.

The mating-type locus, mating, and meiotic machinery and sexual fecundity have been maintained in *C. neoformans* and *C. gattii*, and yet their population structures often exhibit clonality with more limited recombination. At least two mechanisms may constrain sexual reproduction. First, in the serotype A var. *grubii* lineage, fertile **a** and α isolates are present but geographically restricted to sub-Saharan Africa, where active sexual reproduction and recombination occur (Litvintseva et al., 2003). However, this population represents a unique molecular type (VNB) not found elsewhere, and its relationship to worldwide isolates (VNI and VNII) is unclear (Litvintseva et al., 2006). Second, the ability to engage in α-α same-sex mating can contribute to generate diversity and produce infectious spores in an otherwise unisexual population, but it also serves to promote inbreeding, which may generate populations that appear to be clonal.

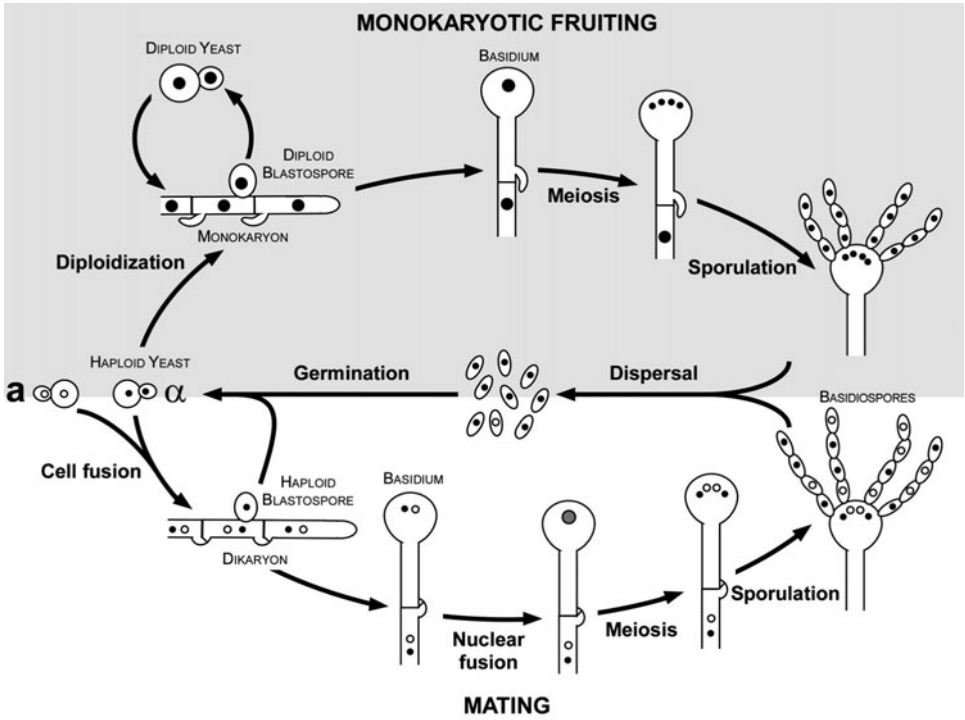

FIGURE 5 Sexual reproduction of *Cryptococcus* involves both **a**-α opposite-sex mating and α-α unisexual mating. The lower panel depicts the well-established heterothallic sexual cycle involving **a** and α mating partners, which fuse to produce a filamentous dikaryon that forms terminal basidia and undergoes meiosis to produce a 1:1 mixture of basidiospores of **a** and α mating types. However, a central conundrum in the field has been the vast disparity in the distribution and prevalence of the two mating types, with α being significantly more common globally. The upper panel depicts a newly discovered sexual cycle involving only α cells, known as monokaryotic fruiting, same-sex mating, or unisexual reproduction. Similar environmental conditions stimulate opposite-sex and same-sex mating. During same-sex mating, α cells can fuse with other α cells or possibly also undergo other forms of diploidization such as endoreplication. Hyphal growth of the resulting diploid isolates is often enhanced and leads to the formation of monokaryotic hyphae with unfused clamp connections, terminal basidia, meiosis, and chains of only α basidiospores. Diploidization can occur early in the differentiation pathway or in some isolates may occur late, possibly only in the basidium, similar to the heterothallic sexual cycle. This unusual homothallic unisexual cycle may have arisen as a consequence of the largely unisexual population or may have driven the success of the α mating type. Some isolates of **a** mating type have been found to undergo same-sex mating. Recent studies reveal that same-sex mating is a quantitative trait controlled by many segregating polymorphic genetic loci. In particular, the *MAT* locus is one of the most significant quantitative trait loci influencing unisexual mating, and the α allele promotes fruiting to a greater extent than the **a** allele, again providing insight into why the α allele may be the predominant form found in nature.

Further studies have the potential to advance understanding of the evolution and pathogenicity of the *Cryptococcus* complex and the roles of sexual reproduction in the evolution and virulence of microbial pathogens.

OPPOSITE-SEX AND SAME-SEX MATING OCCURS IN NATURE

Although the *Cryptococcus* sexual cycle has been known to occur in the laboratory for more than 3 decades (Kwon-Chung, 1975, 1976a), mating has never been directly observed to occur in nature, primarily because the structures produced are microscopic. One indirect proof that mating occurs in the natural population was the discovery that isolates with the unusual AD serotype are in fact diploid or aneuploid strains that in many cases descend from an ancestral mating event between isolates of **a** and α mating type, resulting in the production of **a**ADα and αAD**a** isolates (Cogliati et al., 2001; Lengeler et al., 2001). However, the fact that the α mating type is much more prevalent than the **a** mating type in *C. neoformans* and *C. gattii* populations limits the opportunities for opposite-sex mating to occur in nature. Recently it was discovered that *C. neoformans* can undergo unisexual mating under defined laboratory conditions, especially between α isolates (Lin et al., 2005). However, whether unisexual mating occurs in nature was subject to the same caveat that it has never been directly observed. Recently, natural αADα hybrids that arose via cell-cell fusion between two parental α isolates of different serotypes (A and D) were identified and characterized, providing definitive evidence that unisexual mating also occurs in nature (Lin et al., 2007). A novel truncated allele of the mating-type-specific cell

identity determinant *SXI1*α was discovered to be present in all ADα diploid strains analyzed, including those that arose via opposite-sex mating with an A**a** partner and those that arose via unisexual mating with an Aα partner. This novel *SXI1*α truncation allele may therefore have contributed to enhanced fecundity of the Dα parental isolates, leading to the production of diploid hybrids.

Based on population genetics approaches, the human fungal pathogen *C. gattii* has been found to be undergoing sexual reproduction in tree hollows in Australia, and this occurs within populations that are a mixture of the two mating types, as well as those that are exclusively α mating type (Saul et al., 2008). Eucalyptus trees have been well established as an environmental niche for *C. gattii*, but whether the organism was clonally or sexually reproducing there had been unclear (Campbell and Carter, 2006) until these studies. *C. gattii* is known to undergo mating between strains of opposite mating types in the laboratory (Fraser et al., 2003; Kwon-Chung, 1976b), and yet the natural population is skewed in favor of the α mating type, which limits opportunities for opposite-sex mating in nature. The recent findings that sex between isolates of the same mating type occurs in the laboratory for the sibling species *C. neoformans* and that mating of both *C. neoformans* and *C. gattii* is induced during coculture with plants (Xue et al., 2007) have stimulated interest in when and where unisexual mating might occur in nature. The application of population genetics approaches demonstrated that natural populations of *C. gattii* are undergoing both opposite-sex and same-sex mating in trees in nature, and the balance between the two types of sexual reproduction likely depends on the local availability of mating partners and their mating types (Carter et al., 2007).

Based on population genetics studies, sexual reproduction of *C. neoformans* serotype A VNI molecular type has also been found to occur in trees found throughout India, apparently via unisexual mating in a population that appears to consist exclusively of isolates of α mating type (Hiremath et al., 2008). Previous studies of *C. neoformans* isolated from pigeon guano and human patients suggested that clonal reproduction predominates, but this more recent analysis of a well-defined population from a different environmental niche (trees) found evidence of both clonal reproduction and recombination within the population. Previous studies had revealed that *C. neoformans* (serotype D, var. *neoformans*) can undergo mating between isolates of the same mating type under defined laboratory conditions, and also in nature, in addition to its well-defined sexual cycle involving cells of opposite mating types (Lin et al., 2005, 2006). These studies of natural populations in an arboreal niche in India advance this understanding to provide evidence that same-sex mating is occurring in nature, that this might involve a novel environmental niche (a variety of tree species), and that same-sex mating may therefore also occur in the predominant pathogenic form of the organism (serotype A, var. *grubii*). A challenge that remains is to observe this alternative unisexual cycle under laboratory-defined conditions for serotype A strains of this pathogenic microbe.

Recent studies provide additional evidence that unisexual mating also occurs in the *C. neoformans* var. *grubii* serotype A isolates, which cause the vast majority of infections in AIDS patients worldwide (Bui et al., 2008). Veterinary isolates were collected in the Sydney, Australia, region, with the rationale that because animals have somewhat restricted life spans and typically do not travel long distances, isolates from animals in a restricted geographic region have the potential to define populations of isolates that are undergoing sexual reproduction. All isolates were α mating type and either VNI or VNII molecular type. Loci were found to be in linkage equilibrium, consistent with genetic exchange. Four VNII isolates were found to be diploid, and when these isolates were incubated on acidified V8 mating medium in close confrontation with **a** isolates as a source of mating pheromone, they underwent limited hyphal differentiation and produced basidia, but as yet no sporulation has been observed. Thus, this unique population appears to be recombining and consists exclusively of α isolates, and self-filamentous diploids that are intermediates produced during unisexual mating were found in the population. Importantly, no **a** isolate has ever been reported for the VNII serotype A lineage. Recent studies on ploidy reveal that ~8% of the naturally occurring serotype A population is diploid and the majority are αAAα diploids produced by unisexual reproduction (Lin et al., 2009). Two classes of diploids are apparent: $\alpha A_1A_2\alpha$ isolates that result from fusion of two genetically divergent parents, and $\alpha A_1A_1\alpha$ isolates in which two seemingly identical genomes are present, resulting from either endoreplication or mother-daughter cell fusion. These αAAα diploid intermediates provide further evidence that α–α unisexual reproduction occurs in nature. Taken together, these findings provide evidence that recombination is occurring via unisexual mating and may result in the production of spores and infections in animals in this and other geographic regions.

Taken together, these laboratory and environmental population genetics-based studies provide robust support that both opposite-sex and unisexual reproduction are occurring to impact the diversity and population structure for this pathogenic microbe. An important implication is that unisexual mating may be the route by which infectious propagules are produced in nature, and thus, further studies on both the genetic and genomic control of this novel sexual cycle and the conditions that support this in the lab and in nature are of considerable interest and import. Such studies are likely to illustrate general principles relevant to understanding transitions between modes of sexual reproduction, both in the fungal kingdom and also for other pathogenic microbes (Heitman, 2006) and even with respect to self-fertility in plants and animals.

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Histoplasma capsulatum

CHAD A. RAPPLEYE

Histoplasmosis is one of the most common respiratory fungal infections worldwide, with an estimated 200,000 infections occurring annually in the United States (Ajello, 1971; Goodwin et al., 1981; Rippon, 1988). In regions of endemicity, up to 90% of individuals show serological evidence of prior infection (Edwards et al., 1969). *Histoplasma capsulatum*, a thermally dimorphic fungal pathogen, is the etiologic agent of histoplasmosis. Morphologically, the dimorphism of *Histoplasma* is manifested as a change from hyphal growth at ambient temperatures to a blastic yeast form in mammalian tissues (Kobayashi et al., 1985). This dimorphism also reflects the switch from a saprobic to a parasitic lifestyle (Rippon, 1988).

Histoplasma is acquired from the environment by inhalation of mycelium-produced conidia. Exposure to elevated mammalian body temperature triggers differentiation into budding yeast cells that are effective pathogens of host macrophages. *Histoplasma* can infect both immunocompromised and immunocompetent individuals, with the severity of disease contingent on dose and host immunological status (Rippon, 1988). Among immunocompetent individuals, most infections are self-limiting with the onset of cell-mediated immunity and activation of macrophages (Goodwin et al., 1981; Newman, 2001). However, in some cases, hematogenous spread of fungal cells, particularly in immunocompromised hosts and those receiving immunosuppressive anticytokine therapies, can lead to life-threatening disseminated histoplasmosis (Graybill, 1988; Kaiser, 2007; Tsiodras et al., 2008). Histoplasmosis cases outside regions of endemicity indicate that not all primary infections are completely cleared, resulting in latent disease that can emerge when host immunological function is depressed (Nakelchik and Mangino, 2002; Tseng et al., 2005; Jain et al., 2006; Ashbee et al., 2008).

PHYLOGENETICS AND SPECIES RELATIONSHIPS

Originally, *H. capsulatum* was subdivided into three varieties, based on morphology and the associated disease. In North and South America, *H. capsulatum* var. *capsulatum* was associated with pulmonary disease, while *H. capsulatum* var. *duboisii*, a morphologically larger yeast, was primarily found in cutaneous and subcutaneous disease in Africa. The third variety, var. *farciminosum*, was associated with equine infections. It is now recognized that var. *farciminosum* is not a distinct phylogenetic group but instead characterizes a disease type (Kasuga et al., 2003).

Molecular phylogenetic analyses reveal that *H. capsulatum* comprises at least seven distinct phylogenetic groups associated with different geographical locations. Molecular differences between *Histoplasma* strains have been identified using restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA (Vincent et al., 1986; Spitzer et al., 1990), ribosomal DNA (Spitzer et al., 1989, 1990; Jiang et al., 2000), and the *YPS3* locus (Keath et al., 1989, 1992). These analyses consistently divide *Histoplasma* strains into six major classes (Table 1). Additional polymorphisms have included random amplified polymorphic DNA (Kersulyte et al., 1992; Carter et al., 1996), single nucleotide polymorphisms (Carter et al., 2001), and microsatellites (Carter et al., 1997, 2001), as well as nucleotide sequence differences in the tubulin (*TUB1*), Δ-9 fatty acid desaturase (*OLE1*), ADP-ribosylation factor (*ARF*), and H-antigen genes (Kasuga et al., 1999, 2003). The studies by Kasuga et al. used geographically diverse isolates from both clinical and environmental sources and demonstrated that *Histoplasma* strains can be separated into at least seven major clades, which have been given designations that reflect their geographical distribution (Kasuga et al., 2003).

The phylogeographical analysis of *Histoplasma* supports the earlier RFLP classification scheme. A harmony of these designations is presented in Table 1. In North America, two phylogenetic species exist: NAm 1 (RFLP class 1) and NAm 2 (RFLP class 2). The genomes from representative clinical isolates of NAm 1 and NAm 2 have been completely sequenced, as well as a clinical isolate from the Panamanian clade (RFLP class 3). The vast majority of molecular studies on *Histoplasma* have used strains from these three groups. RFLP class 4 is represented by only a single isolate from Florida, and its utility in the classification of *Histoplasma* strains is relatively minor. In Latin America, two phylogenetic groups, LAm A (RFLP class 5) and

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TABLE 1 Classification of *Histoplasma* strains

Phylogenetic group[a]	RFLP class[b]	Chemotype[c]	Type strain[d]	Other major strains[e]	*Ura5*Δ background[f]
NAm 1	Class 1	2 (α-Glucan$^+$)	WU24	Downs (ATCC 38904); UCLA531S	
NAm 2	Class 2	1 (α-Glucan$^-$)	G217B (MYA-2455)	G222B (ATCC 26034); UCLA 505 (ATCC 28122)	WU15
Panama	Class 3	2 (α-Glucan$^+$)	G186A (MYA-2454)	G184A (ATCC 26027); G184B (ATCC 26028); G186B (ATCC 26030)	WU8
	Class 4			FLS1	
LAm A	Class 5	2 (α-Glucan$^+$)			
LAm B	Class 6	2 (α-Glucan$^+$)			
Africa		2 (α-Glucan$^+$)		(*H. capsulatum* var. *duboisii*); RV26821 (ATCC 32281)	

[a]Data from Kasuga et al., 1999, 2003.

[b]Spitzer et al., 1989; Keath et al., 1992.

[c]Domer, 1971; Kanetsuna et al., 1974; Reiss, 1977.

[d]Strains commonly used in molecular genetic studies and which have sequenced genomes. ATCC accession numbers are given in parentheses.

[e]Other popular strains used in research studies. ATCC accession numbers are given in parentheses.

[f]Uracil auxotroph strains based on the type strains for genetic manipulations with Ura5$^+$-based episomes.

LAm B (RFLP class 6), are present in addition to those isolates from Panama. Although the geographical distributions overlap between some groups, the deep branching of the phylogenetic trees suggests that these groups do not recombine with each other in nature (Kasuga et al., 2003). Considerable diversity exists within the NAm 2 group, indicating a recombining population within this clade (Carter et al., 1996, 1997). Nevertheless, separate subclasses exist within the NAm 2 group, which reflect geographical separations (Carter et al., 2001). *H. capsulatum* var. *duboisii* is equivalent to the African phylogenetic species.

Phylogenetic separations may also reflect differences in the pathogenesis of the respective strains. Some studies have shown a correlation between NAm 1 strains and a human immunodeficiency virus (HIV)-positive status of the host, suggesting that NAm 1 might represent a strain of *Histoplasma* with reduced virulence; the majority of clinical isolates in North America are NAm 2 strains, but the few NAm 1 isolates have come primarily from AIDS patients (Medoff et al., 1986; Vincent et al., 1986; Spitzer et al., 1990). It is unclear whether this bias represents differences in the underlying environmental prevalence of the two North American types or if NAm 1 strains are unable to cause significant disease in non-AIDS-afflicted individuals. The Downs NAm 1 isolate has a higher 50% lethal dose for mice than do NAm 2 or Panamanian strains and has reduced growth at elevated temperatures of 37 to 41°C (Medoff et al., 1986). Three of three NAm 1 strains from the St. Louis area were isolated from AIDS patients, whereas non-AIDS patients presented only with NAm 2 *Histoplasma* strains (Spitzer et al., 1990). These three NAm 1 isolates showed temperature-sensitive growth similar to that of the Downs isolate. However, these findings are based on a relatively small sample set and an additional NAm 1 strain has been isolated from a HIV-negative individual, which calls into question the conclusion that NAm 1 strains represent a lower virulence group (Jiang et al., 2000). No similar bias for HIV-positive individuals has been found for the two Latin American strain groups (Muniz et al., 2001; Kasuga et al., 2003). In contrast to the primarily respiratory manifestation of histoplasmosis caused by North American strains, Latin American and especially African strains are associated with a greater degree of cutaneous lesions, indicating differences in the underlying pathogenesis between these groups (Rippon, 1988; Karimi et al., 2002). In a murine model of acute pulmonary infection, LAm A and LAm B strains caused higher mortality than a NAm 2 strain (Durkin et al., 2004). However, in a low-dose inoculum model of chronic infection, only the NAm 2 strain showed significant persistence and outgrowth in the lung (Durkin et al., 2004). This may suggest a greater propensity for reactivation histoplasmosis among NAm 2 infections.

Histoplasma strains have also been classified according to chemical differences in the yeast cell wall. The various serotypes could be grouped by different cell wall solubility in NaOH (Pine and Boone, 1968) and by chitin and polysaccharide composition (Domer, 1971). This led to the classification of strains as either chemotype 1 or chemotype 2 (Domer, 1971). Further investigations demonstrated that chemotype 2 cell walls contain glucan polysaccharides with α-(1,3)-glycosidic bonds that are lacking in chemotype 1 yeast (Kanetsuna et al., 1974; Reiss, 1977; San-Blas et al., 1978). The chemotype 1 yeasts that lack α-(1,3)-glucan are now recognized as those of the NAm 2 class (Table 1). Yeasts from the remaining phylogenetic groups, including the other North American class, all have cell walls containing α-(1,3)-glucan. These chemotype 2 strains are more similar in composition to the yeast cell walls of the other dimorphic fungal pathogens (e.g., *Blastomyces* and *Paracoccidioides*). Furthermore, biosynthesis of α-(1,3)-glucan contributes to *Histoplasma* pathogenesis (see below).

GENETICS AND MOLECULAR GENETICS

The Genome(s) of *Histoplasma*

The genomes of three *Histoplasma* strains representing NAm 1, NAm 2, and the Panamanian phylogenetic groups have been sequenced (http://www.broad.mit.edu and http://www.genome.wustl.edu). *Histoplasma* is haploid, with an average genome GC content of 42 to 46% (Bawdon et al.,

1972; Carr and Shearer, 1998). Electrophoretic studies estimate at least seven chromosomes for Downs (NAm 1), three chromosomes for NAm 2, and four chromosomes for strains from the Panama group (Steele et al., 1989). The genome size differences between phylogenetic groups are largely due to the amount of repetitive DNA based on reassociation kinetics (Carr and Shearer, 1998). The current genome sequence assemblies for the NAm 1 and Panama type strains are 30.6 and 30.5 Mb, respectively. In contrast, the NAm 2 genome is substantially larger, at 41 Mb, but 31% of the NAm 2 genome is comprised of repetitive DNA compared to only 11% in the NAm 1 genome. The nature of the repetitive DNA includes a substantial amount of mobile DNA insertions, including retrotransposons and multiple copies of a *Histoplasma* crypton (Goodwin et al., 2003). Thus, the core, nonrepetitive genome of *Histoplasma* is approximately 26 to 28 Mb and encodes an estimated 9,000 to 11,000 genes, based on gene prediction models. An oligonucleotide-based transcriptional microarray has been constructed from the gene predictions from the NAm 2 and Panama type strains and is maintained at the Genome Sequencing Center at Washington University, St. Louis, MO.

Mating

Histoplasma has a binary heterothallic mating system, which facilitates recombination among *Histoplasma* populations in the environment. This mating system operates in the mycelial phase between strains designated (−) and (+) mating types and results in production of eight globular ascospores, which inherit one or the other mating type (Kwon-Chung, 1972a, 1972b, 1973). The proportion of (−) and (+) mating types in a particular environmental location varies considerably with some sites at or near homogeneity (Kwon-Chung et al., 1974; Gaur and Lichtwardt, 1980). The (−) and (+) mating types are both virulent in mice. Although some studies have suggested that (−) mating-type strains may convert to the pathogenic yeast form more easily than (+) strains (Kwon-Chung, 1973; Kwon-Chung et al., 1974), other studies found no difference in conversion rate in vitro (Gaur and Lichtwardt, 1980) and both mating types can be recovered after passage through mice (Kwon-Chung, 1973). Nevertheless, the prevalence of the (−) mating type among clinical *Histoplasma* isolates suggests that mating type may influence pathogenesis (Kwon-Chung et al., 1974, 1984).

The complete sequencing of *Histoplasma* genomes has enabled the molecular characterization of the mating locus. Mating type is specified by one of two different alleles at the *MAT* locus (Table 2). In *Histoplasma*, the *MAT* locus lies between the *COX13* and *SLA2* genes (Fraser et al., 2007). The 5.4- to 5.7-kb *MAT1-1* allele encodes an α-box domain transcription factor that specifies the (+) mating type, and the 5.0-kb *MAT1-2* allele encodes a high-mobility-group (HMG)-domain protein that confers the (−) mating type (Bubnick and Smulian, 2007; Fraser et al., 2007). *Histoplasma* produces mating pheromones that are chemically similar to the **a** and α mating pheromones of *Saccharomyces*, and treatment of (+) mating type cells with the α-type pheromone or (−) cells with the **a**-type pheromone increases expression of the *MAT* locus (Bubnick and Smulian, 2007). Although laboratory-passaged strains of *Histoplasma* rapidly lose mating competence, thereby preventing classical genetic crosses (Kwon-Chung, 1973; Kwon-Chung et al., 1974), continued definition of the mating pathway may open up prospects to restore mating ability through manipulation of genes downstream of mating-type specification.

Molecular Genetic Tools

Transformation and Transgene Expression

The absence of classical genetics has spurred the development of molecular genetic tools for the analysis and manipulation of *Histoplasma*. *Histoplasma* yeast cells can be transformed with purified DNA either through chemical treatment with PEG-lithium acetate or by electroporation (Worsham and Goldman, 1990; Woods and Goldman, 1993). Key to the efficient transformation of *Histoplasma* yeast and maintenance of the transforming DNA as an extrachromosomal element is the use of linear DNA molecules with telomeric sequence repeats (GGGTTA) at the ends to mimic the eukaryotic chromosome structure (Woods and Goldman, 1993; Woods et al., 1998). Selection for transforming DNA is provided through expression of the *Escherichia coli* hygromycin phosphotransferase gene (*hph*) or the fungal *URA5* gene in a *ura5* auxotrophic background (Woods and Goldman, 1993). Under selective pressure, linear plasmids are maintained, but their copy number is unknown. Use of *URA5* as the selection marker has the added utility of allowing for maintenance of plasmids during growth within mammalian hosts (Retallack et al., 1999). A final method of transformation of *Histoplasma* exploits the ability of *Agrobacterium tumefaciens* to transfer an engineered T-DNA element into fungal cells (Sullivan et al., 2002). In contrast to transformation with linear telomeric plasmids, *Agrobacterium*-mediated transformation results in chromosomal integration of the transforming DNA.

Both heterologous and native transgenes can be expressed from transforming DNA molecules using fungal promoters. Linear telomeric plasmids harboring colorimetric (*lacZ*) or fluorescent (*gfp*) reporter genes provide visual and quantitative assessment of gene expression (Patel et al., 1998; Kugler et al., 2000b). Various promoters have been used to express transgenes in *Histoplasma*, including *Aspergillus gpd*, *Podospora URA5*, and a handful of endogenous *Histoplasma* promoters: *TEF1* (Tian and Shearer, 2002), *URA5* (Woods et al., 1998), and *H2B* (Rappleye et al., 2004). A few promoters provide

TABLE 2 *Histoplasma* mating types

Mating type	Allele	Encoded transcription factor	Responds to pheromone	Strain isolates
(+)	*MAT1-1*	HMG domain	α-Type	G217B
				WU24
				T-4-2
				UH1
(−)	*MAT1-2*	α-box domain	**a**-Type	G186A
				T-3-1
				VA1

regulated transgene expression; the *CBP1* promoter restricts expression specifically to the pathogenic yeast phase (Kugler et al., 2000a, 2000b) and the promoter for the *CRP1* copper efflux pump can be induced by exogenous application of copper (Gebhart et al., 2006).

Gene Deletion

Demonstration that a gene is linked to a process under study requires methodology to delete or deplete gene functions (Fig. 1). Although the dominance of nonhomologous recombination in *Histoplasma* frustrates allelic replacement strategies, a two-step method has been developed to generate gene deletions (Sebghati et al., 2000; Magrini and Goldman, 2001). The solution is to (i) provide a large enough population of mitotic events that the rare homologous recombination event is realized and (ii) employ a selection scheme that permits isolation of the recombinant from the bulk population of nonrecombinants. In the first step, *Histoplasma* $Ura5^-$ auxotrophic yeast cells are transformed with a $Ura5^+$ linear telomeric plasmid containing an *hph* selection marker that interrupts the targeted gene coding sequence. The disruption allele is flanked by 1 to 2 kb of upstream and downstream sequence homologous to the target to provide a recombination substrate. A large clonal population of individual transformants is passaged multiple times. The second step employs positive and negative selections to enrich for the desired double-crossover event. Hygromycin resistance selects for the disruption allele, while the use of 5-fluoroorotic acid selects against *URA5* and maintenance of the episomal plasmid. This process was pioneered in the generation of the *CBP1* deletion (Sebghati et al., 2000). In practice, only a portion of the isolated clones harbor the deletion and allelic replacement success has been achieved in the Panama background only (G186A) despite numerous attempts with the NAm 2 strain G217B (Rappleye et al., 2004; Marion et al., 2006; Hwang et al., 2008).

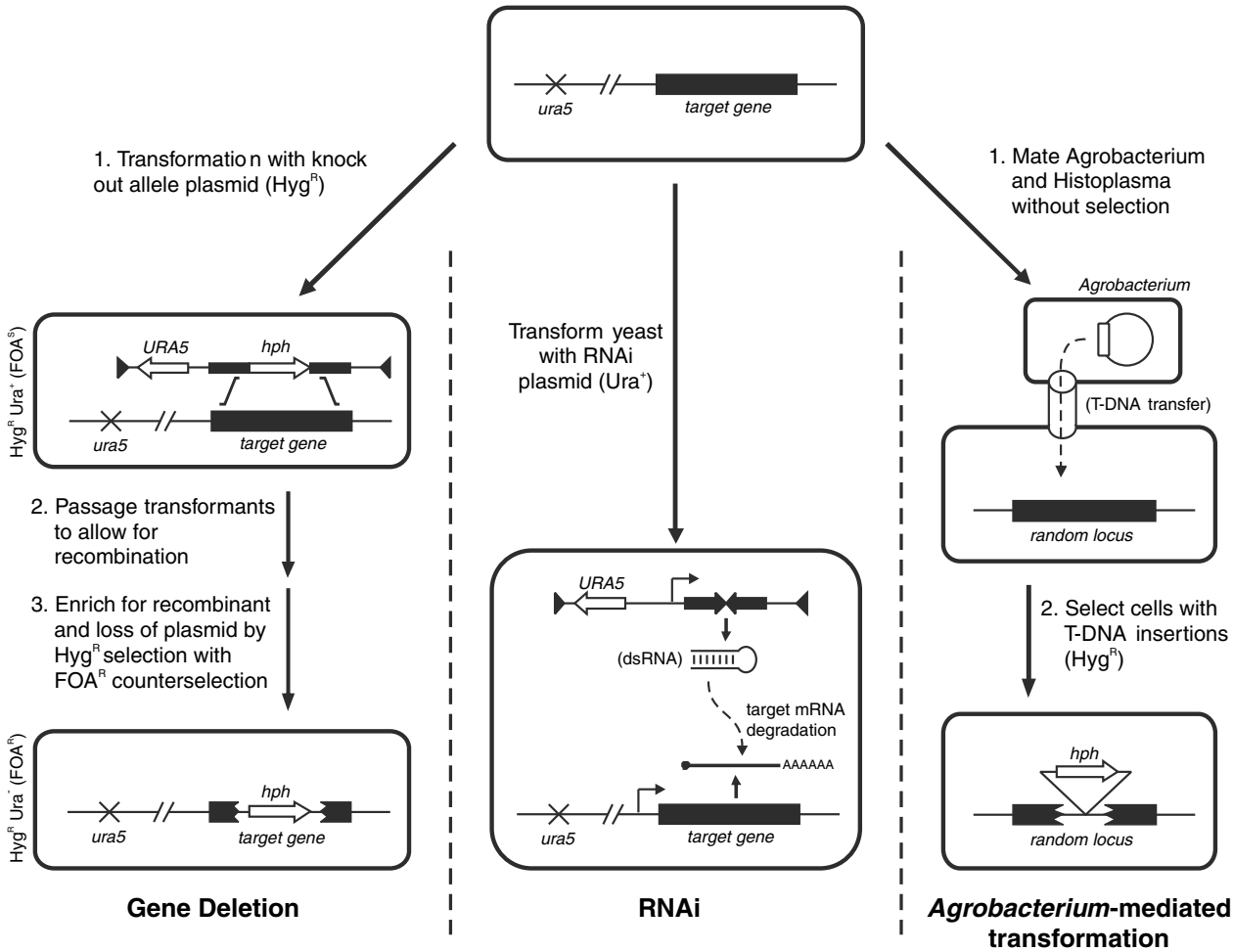

FIGURE 1 Gene inactivation methodology for *H. capsulatum*. Three methods have been developed for inactivating gene functions in *Histoplasma* cells. Deletion of a target gene is accomplished through allelic replacement with a disruption allele. To enrich for recovery of cells in which the desired homologous recombination event has occurred, positive and negative selections are employed. RNAi can be used to deplete gene functions posttranscriptionally by transformation of *Histoplasma* yeast with a linear plasmid containing inverted copies of the target locus. Subsequent transcription leads to formation of double-stranded RNAs that trigger the RNAi effect. Genes can also be disrupted by *Agrobacterium*-mediated transfer of a T-DNA element that inserts randomly into *Histoplasma* chromosomes.

RNAi

In light of the difficulty and the laborious process required to generate deletion alleles, RNA interference (RNAi) has emerged as a more efficient means to deplete gene functions. Gene silencing through RNAi is triggered by double-stranded RNA molecules homologous to the targeted gene. In *Histoplasma*, transcription of a transgene composed of inverted copies of the gene of interest generates the double-stranded RNA molecule. This RNA molecule is recognized by the endogenous RNAi machinery and cleaved to produce small interfering RNAs (Rappleye et al., 2004). The small interfering RNAs guide the RNAi machinery to the cognate endogenous mRNA, resulting in degradation of the native transcript. Induction of RNAi in *Histoplasma* relies upon a single transformation step and provides posttranscriptional silencing of a gene of interest. While RNAi cannot completely silence a targeted gene like deletion alleles can, RNAi is simpler and quicker and is effective in both Panama and NAm 2 strains. Genes silenced by RNAi to date include *AGS1* (Rappleye et al., 2004), *YPS3* (Bohse and Woods, 2007b), *DRK1* (Nemecek et al., 2006), *UGP1* (Marion et al., 2006), and *RYP1* (Nguyen and Sil, 2008).

Insertional Mutagenesis

Agrobacterium-mediated transformation is employed as an insertional mutagen that facilitates forward genetic screens in *Histoplasma*. For transformation, *Histoplasma* yeast cells are cocultured with *Agrobacterium tumefaciens* in the presence of acetosyringone to induce the *Agrobacterium* type IV secretion system (Sullivan et al., 2002). Transfer of T-DNA (transferred DNA) elements from the *Agrobacterium* tumor-inducing plasmid that contain an appropriate selectable marker for fungi allows the isolation of *Histoplasma* transformants, of which 80% contain a single T-DNA insertion that can be efficiently mapped using molecular techniques. This process has been used to identify new genes involved in α-glucan biosynthesis (Marion et al., 2006) and factors required for the transition to the yeast phase (Nguyen and Sil, 2008).

DIMORPHISM

Mycelial Phase

Histoplasma exists as a multicellular saprobic mycelium in soil environments and in laboratory culture at temperatures below 32°C (Rippon, 1988). The mycelium is composed of septate hyphae, which grow by apical extension as well as branching. The mycelial form produces microconidia (2 to 5 μm in diameter) and larger macroconidia (8 to 14 μm in diameter) which function in aerosol dispersion of *Histoplasma* (Anderson and Marcus, 1968). Microconidia in particular are small enough to reach the alveoli of a mammalian host. The number and type of conidia produced depend on the strain and the growth medium, but in general microconidia predominate. Both micro- and macroconidia can have tubercles on the outer surface of the conidial cell wall; these protrusions on macroconidia are often visible by light microscopy (Pine and Webster, 1962; Garrison and Boyd, 1977, 1978). On Sabouraud's medium, *Histoplasma* mycelia generate two different colony types (Berliner, 1968). The "A" type (for "albino") consists of coarse, white hyphae that produce smooth macro- and microconidia initially but become nonsporulating upon continued passage in culture. The second colony type, "B" (for "brown"), is formed from sparse, narrow, pigmented hyphae which produce copious amounts of tuberculated macroconidia. The specific colony type produced by *Histoplasma* isolates is designated by a suffix "A" or "B" appended to the strain name (e.g., G186A). Microscopically, mycelia contain a normal complement of eukaryotic organelles and cytosolic constituents. The hyphal cell wall of *Histoplasma* is comprised primarily of β-glucans, chitin, galactomannans, and protein (Domer et al., 1967; Kanetsuna et al., 1974; Kanetsuna, 1981).

Environmental isolates of *Histoplasma* have been obtained from soils that are nearly always associated with the guano of birds (starlings, pigeons, and chickens) and bats (Emmons, 1950, 1958; Ajello, 1964; Campbell, 1965; Cano and Hajjeh, 2001). Slight acidity represents an additional factor correlated with the presence of *Histoplasma* in soil (Zeidberg et al., 1955; Emmons, 1956). The Ohio and Mississippi River basins in the United States, where *Histoplasma* is endemic, significantly overlap with the distribution of starling and blackbird roosts, suggesting these avian species contribute to the abundance of *Histoplasma* in these locations (Smith and Furcolow, 1964; Tosh et al., 1970; Rippon, 1988). The higher body temperature of fowl hinders infection of the birds themselves with *Histoplasma*. In contrast, studies have shown that bats are infected with *Histoplasma* and that migratory bat species may contribute to the geographical distribution of *Histoplasma* groups (Shacklette et al., 1962; Emmons et al., 1966; Taylor et al., 2005). Most outbreaks of histoplasmosis can be traced back to environmental or human disturbance of *Histoplasma*-infested soils, causing airborne release of conidia.

Molecular characterization of the mycelial phase of *Histoplasma* is limited. Genes enriched or unique to the mycelial phase have been identified through a subtractive cDNA library approach and through microarray-based transcriptional profiling (Tian and Shearer, 2001; Hwang et al., 2003). From the microarray, genes showing highly enriched expression in the mycelial phase include a tyrosinase homolog (*TYR1*), which may function in melanin production; *MPS1* (mycelial-phase-specific protein 1) of unknown function; and homologs of genes involved in the conidiation process (Hwang et al., 2003). Catalase A is also expressed specifically by mycelial-phase *Histoplasma* (Johnson et al., 2002). Tian et al. identified *MS8* and *MS88* as two mycelial-phase-specific genes (Tian and Shearer, 2001). *MS8* is highly expressed in mycelia and has become a standard marker for mycelium-specific expression. Of all these mycelial-phase-enriched genes, functional studies have been done only with *MS8* (Tian and Shearer, 2002). Ectopic expression of *MS8* in the yeast phase did not significantly affect yeast cells. Loss of *MS8* through gene deletion similarly has no effect on yeast cells, but without *MS8* function in the mycelial phase, hyphal cells become shorter and thicker, indicating that *MS8* may regulate hyphal cell structure.

Yeast Phase

The yeast phase of *Histoplasma* is the morphological form found in infected tissues and is the phase devoted to pathogenesis. *Histoplasma* yeast cells are uninucleate and replicate by budding, whereby the switch from isotropic to polarized growth produces the daughter bud. *Histoplasma* yeast cells are small, only 2 to 5 μm in diameter, except those of the African group (var. *duboisii*), which are typically 12 to 15 μm in diameter. *Histoplasma* has a characteristic narrow neck between the mother yeast cell and the daughter bud. The yeast cell wall contains primarily β-glucans (mostly β-1,3-linkages), chitin, and smaller amounts of galactomannan and protein (Domer et al., 1967; Pine and Boone, 1968; Domer, 1971; Kanetsuna et al., 1974).

The cell wall of chemotype 2 strains contains the additional α-glucan polysaccharide and has less chitin than chemotype 1 strains (Domer, 1971; Kanetsuna et al., 1974). Ultrastructural studies reveal that these cell wall constituents are organized into a multilayered structure with chitin and β-glucan fibrils comprising the innermost layer, surrounded by an outer layer of short thick fibers corresponding to α-glucan polysaccharides (Kanetsuna et al., 1974; Rappleye et al., 2007). Susceptibility of the cell wall to enzymatic digestion and hydrolysis provides further support for this organization, with inner-layer polysaccharides released following removal of the outer α-glucan (Domer, 1971; Reiss, 1977). At least some cell wall antigens are associated with β-glucan and/or chitin, since enzymatic removal of α-glucan results in greater antigen reactivity of the remaining cell wall. Furthermore, β-glucanase solubilized fractions from chemotype 1 strains are immunoreactive (Reiss et al., 1977). The β-glucan/chitin structure dictates the shape of yeast cells, since removal of α-glucan does not affect yeast cell shape (Kanetsuna, 1981) but enzymatic or nonenzymatic digestion of the remaining β-glucan and chitin containing cell wall yields spherical protoplasts (Berliner et al., 1972; Carbonell et al., 1972). Additional molecular details that pertain to the yeast phase are discussed below in "Pathogenesis."

Transition to the Yeast Phase

Morphological Change

When subjected to a temperature of 37°C, germinating *Histoplasma* conidia or hyphal cells undergo a morphological conversion to produce yeast phase cells. Germinating microconidia at 37°C generate a bud-like protrusion that can (i) continue to elongate forming a germ tube, (ii) enlarge to form a bulbous cell termed a yeast mother cell (YMC), or (iii) directly give rise to a yeast-like cell (Pine and Webster, 1962; Garrison and Boyd, 1978). In the first case, the elongating germ tube culminates in the formation of a short septate mycelium, which can subsequently give rise to yeast cells via mycelium-to-yeast transition mechanisms described below. In the second pathway, the conidium-YMC complex has a continuous cytoplasm and develops single or multiple buds along the YMC periphery that enlarge to form yeast daughter cells. In these two microscopic studies of conidial germination, neither group observed conversion of macroconidia into yeast cells, which might indicate that the germination of macroconidia into yeast may proceed strictly through a hyphal transitional state.

The morphological transition of hyphae to yeast cells is described by a time-lapse microscopic analysis of hyphal fragments at 37°C (Pine and Webster, 1962). Yeast cells are ultimately derived by budding from a YMC-like structure, the formation of which may occur through various pathways. Individual hyphal cells may swell to form a monilial chain, and separation of these cells produces a cluster of YMCs. Alternatively, unswollen hyphal cells, either terminal or interstitial, can bud to form a YMC that may or may not remain attached to the hyphal cell during the generation of daughter yeast cells. Although mycelial-phase cells can convert to the yeast phase in culture, natural infection by *Histoplasma* most often occurs by inhalation of conidia. The transition mechanisms that operate in vivo remain unknown, but the fact that conidia can produce yeast cells through a hyphal intermediate indicates that continued investigation of the mycelium-to-yeast conversion is warranted.

Signals for Conversion

The complete transition from mycelium to yeast has two requirements: elevated temperature and the presence of sulfhydryl compounds. *Histoplasma* is thermally dimorphic, with elevated temperature providing the inducing signal for transition to the yeast phase. Temperatures above 30°C are sufficient to signal conversion, with 37°C being optimal (Scherr, 1957; Pine and Webster, 1962). As expected, increased temperature causes an immediate heat shock response, which includes transcriptional upregulation of the heat shock protein *HSP70* (Shearer et al., 1987). Whether the heat shock response is required for the yeast phase remains to be determined, but the correlation between temperature sensitivity of the Downs strain and lower *HSP70* synthesis levels compared to NAm 2 strains suggests that the heat shock response may have a causal effect (Lambowitz et al., 1983; Caruso et al., 1987). The temperature shift signals hyphal cells through an unknown mechanism to enter the first of three stages required for complete conversion to the yeast phase (Fig. 2) (Maresca et al., 1981).

The first stage of the mycelium-to-yeast transition is marked by a pronounced decrease in cellular respiration (Maresca et al., 1981; Lambowitz et al., 1983). Respiration in *Histoplasma* consists of two terminal oxidase pathways, a cytochrome system and an alternative oxidase, both of which are functional in the mycelial and yeast phases (Maresca et al., 1979; Johnson et al., 2003). The uncoupling of oxidative phosphorylation in stage I causes a progressive decline in cellular ATP levels (Lambowitz et al., 1983). RNA and protein synthesis decrease during this stage, as well as intracellular amino acid concentrations (Cheung et al., 1974; Maresca et al., 1981). Cyclic AMP (cAMP) levels, normally high in mycelial-phase cells, also decline during stage I (Maresca et al., 1977). As cAMP is a well-known signaling molecule, it is possible that cAMP-dependent signaling cascades directly influence the transition process. In support of this, increasing cAMP levels of yeast cells (that normally have low cAMP concentrations) by addition of exogenous cAMP or cAMP phosphodiesterase inhibitors causes yeast to convert to mycelia at 37°C (Maresca et al., 1977; Sacco et al., 1981). Soon after the switch from 25 to 37°C, a cystine reductase is induced, which may contribute to the generation of reduced sulfhydryl compounds (Maresca et al., 1978). By 24 to 40 h, transitioning cells enter the second stage, essentially a dormant period typically lasting anywhere from 32 to 96 h.

Exit from stage II and completion of conversion to the yeast phase require low-molecular-weight sulfhydryl compounds or a reducing environment. Early studies showed that sulfhydryl-containing compounds (e.g., cysteine) are necessary for conversion of mycelia to yeast cells and growth of yeast at 37°C (Salvin, 1949; Pine, 1954; Scherr, 1957), but the sulfhydryls are not merely a nutritional requirement for the transition. In the absence of exogenous sulfur-containing compounds, electrolytically lowering the redox potential is sufficient to promote conversion to the yeast phase, even when only a transient temperature shift is applied (Rippon, 1968; McVeigh and Houston, 1972). This suggests that the sulfhydryl-requirement contributes to conversion to the yeast phase by supplying reducing potential. While the precise role of cysteine or other sulfhydryls in stage II remains unknown, cysteine stimulates respiration and shortens the duration of stage II, possibly by inducing respiratory shunt pathways (Maresca et al., 1977; Sacco et al., 1983). A sulfhydryl-blocking agent,

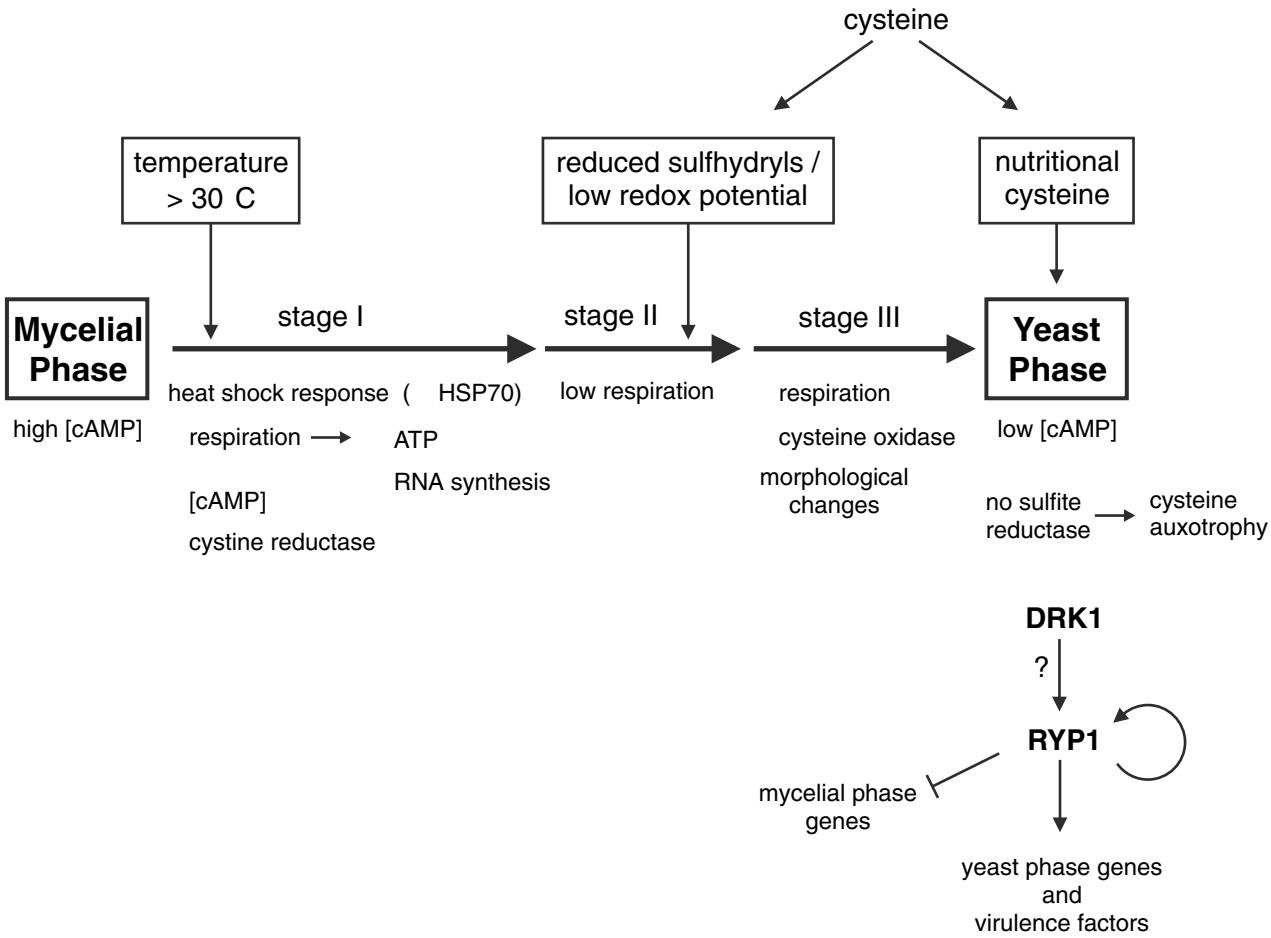

FIGURE 2 Transition of mycelial phase to yeast phase of *H. capsulatum*. Two signals are required for the complete transition of mycelial phase to the yeast phase: elevated temperature and cysteine. Cysteine plays a twofold role by providing reduced sulfhydryls/low redox potential necessary to complete stage II as well as supplying organic sulfur to complement the cysteine auxotrophy of yeast cells. Additional characteristics of stages in the conversion process are presented. The DRK1 kinase and RYP1 DNA-binding protein promote differentiation to the yeast phase and control expression of the yeast phase regulon including transcription of *Histoplasma* virulence factors.

p-chloromercuriphenylsulfonic acid (PCMS), irreversibly blocks the mycelium-to-yeast transition between stages 2 and 3, effectively preventing the conversion to the yeast phase (Maresca et al., 1977).

In addition to its role as a sulfhydryl source for the transition process, cysteine is also necessary to maintain cells in the yeast phase (Salvin, 1949; McVeigh and Houston, 1972). Yeast cells, but not mycelia, require cysteine for growth due to the absence of sulfite reductase specifically in yeast phase cells (Stetler and Boguslawski, 1979). Sulfite reductase, which reduces sulfite to sulfide, is involved in the assimilation of oxidized sulfur into cysteine and methionine biosynthetic pathways. The onset of stage III of the transition process is characterized by increased expression of cysteine oxidase, which produces cysteinesulfinic acid, an intermediate of cysteine metabolism (Maresca et al., 1981; Kumar et al., 1983). However, the role of this cysteine oxidase in the cysteine auxotrophy of yeast or in phase transition has not been further clarified. Requiring that two conditions must be met for transition to, and maintenance of, the yeast phase (temperature and sulfhydryls/redox potential) may ensure that differentiation to the yeast phase occurs only within the mammalian host environment (Maresca and Kobayashi, 1989).

Molecular Control of Dimorphism

Genetic approaches have identified some of the regulatory components that govern conversion of *Histoplasma* to the yeast phase. In both *Histoplasma* and the closely related dimorphic fungal pathogen *Blastomyces dermatitidis*, a histidine kinase encoded by the *DRK1* gene is necessary for conversion of cells to the yeast phase and for expression of yeast phase genes (Nemecek et al., 2006). The DRK1 kinase is a functional homolog of the *Saccharomyces cerevisiae* Sln1 protein, which controls responses to environmental stimuli. The *Histoplasma RYP1* gene encodes a DNA-binding protein that controls expression of the majority of the genes that constitute the yeast phase regulon (Nguyen and Sil, 2008). *Histoplasma* RYP1 is homologous to the *Candida* Wor1 protein, which controls differentiation to, and maintenance of, the opaque developmental state (Zordan et al., 2006). Consistent with its role as a yeast phase regulator, RYP1 shows higher expression in yeast phase cells than in mycelia (Nguyen and Sil, 2008). RYP1 influences its own expression

levels through binding to its promoter, creating an autofeedback loop. The transcriptional profile of the *ryp1* mutant at 37°C is essentially that of a wild-type cell in the mycelial state, indicating that *RYP1* encodes a master regulator of yeast phase genes and the yeast phase differentiation state. Both DRK1 and RYP1 are components of the temperature-induced mechanism underlying transition to the yeast phase, since the respective mutants grow as mycelia at 37°C on cysteine-containing media. A speculative model based on the similar *ryp1* and *drk1* mutant phenotypes suggests that DRK1 may act in sensing and/or transducing the temperature signal to RYP1, which in turn controls repression of mycelial-phase genes and activation of the yeast phase regulon.

PATHOGENESIS

Histoplasma as an Intracellular Pathogen

Histoplasma is found predominantly within macrophages during infection, occupying an intracellular niche permissible for yeast growth and replication (Fig. 3). In addition to macrophages, polymorphonuclear leukocytes (PMNs), dendritic cells, and even epithelial cells can serve as host environments for *Histoplasma* yeast. As lung surfactant proteins SP-A and SP-D can directly kill *Histoplasma* yeast, residence within phagocyte cells provides a protected environment from surfactant (McCormack et al., 2003). The outcome of the encounter between *Histoplasma* cells and

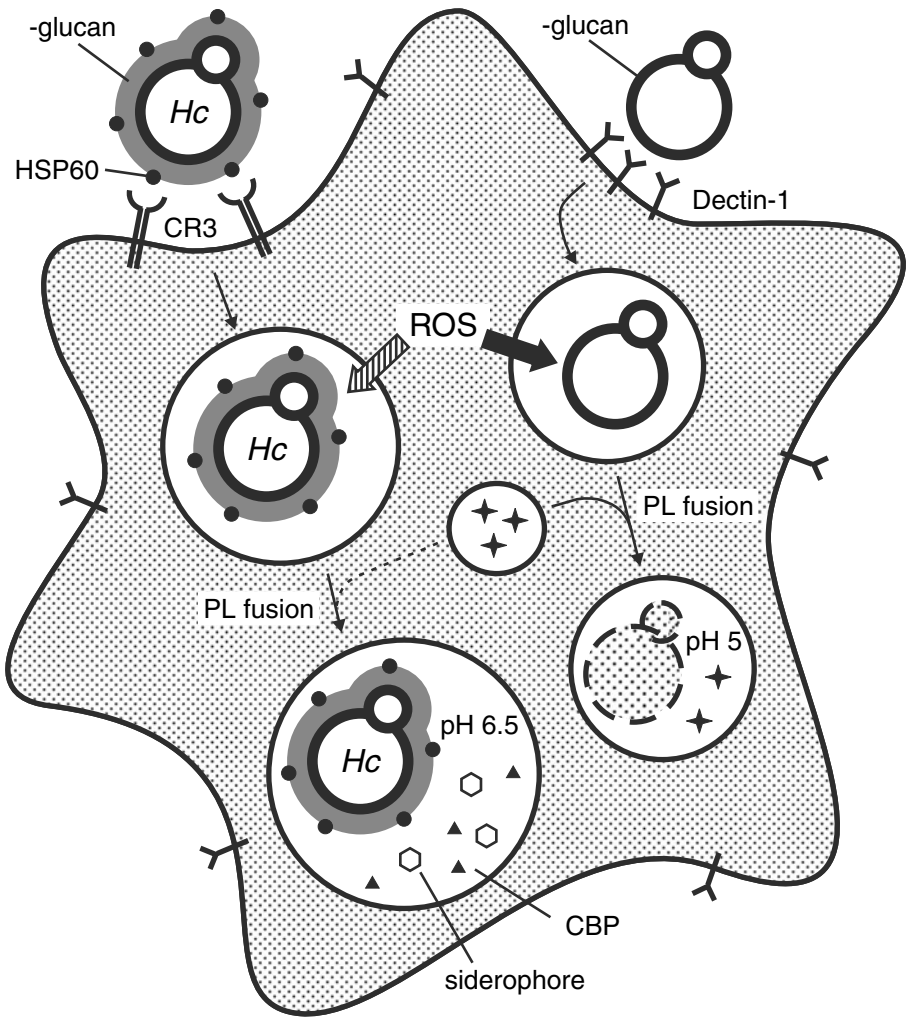

FIGURE 3 Intracellular pathogenesis of *H. capsulatum*. *H. capsulatum* yeast cells (Hc) bind to macrophage CD18 family complement receptors (CR3) via surface-localized HSP60. Cell wall β-glucans of nonpathogenic yeast are recognized by host Dectin-1 surface receptors. In contrast, the cell wall α-glucan layer masks *Histoplasma* from detection. Uptake of *Histoplasma* yeast by phagocytes can trigger production of reactive oxygen compounds (ROS) depending on host species and opsonization, but *Histoplasma* is not killed. In some macrophage cells, *Histoplasma* yeast cells impair phagolysosomal fusion (PL fusion). Phagosomes containing nonpathogenic yeast become acidified and readily fuse with lysosomes, leading to destruction of the nonpathogenic yeast cells. For *Histoplasma*, in situations where phagolysosomal fusion occurs, *Histoplasma* yeast cells escape lysosomal degradation by actively maintaining a luminal pH of >6. In the phagosome or phagolysosome, *Histoplasma* yeast cells secrete iron-chelating siderophores and a CBP, which further promote *Histoplasma* virulence.

phagocytes depends initially on which receptors are engaged and the nature of the interacting molecules between the host and pathogen.

Entry into Host Cells

Histoplasma microconidia and yeast bind to phagocyte complement receptors (CD18-family integrins CR1, CR3, and CR4) to facilitate internalization by phagocytes (Bullock and Wright, 1987; Newman et al., 1990; Schnur and Newman, 1990). Adhesion and internalization into phagocytes does not depend on prior opsonization of fungal cells with complement proteins. The fungal ligand for this adhesion is the *Histoplasma* heat shock protein HSP60 (Long et al., 2003). Although normally a cytosolic protein, some HSP60 is present on the yeast cell surface (Long et al., 2003), and an epitope between residues 214 and 484 of HSP60 mediates the association of *Histoplasma* yeast with CR3 (Habich et al., 2006). It is unknown whether conidia also utilize HSP60 for binding to phagocytes or if they employ a different adhesin. Other phagocyte surface receptors, such as the mannose receptor and the Fc immunoglobulin receptor, do not contribute significantly to binding of *Histoplasma* yeast (Bullock and Wright, 1987).

Normally, phagocytosis of microbes is associated with production of reactive oxygen compounds, yet *Histoplasma* can prevent the generation of, or contend with, these microbicidal products. In the encounter with murine macrophages, *Histoplasma* yeast cells do not trigger macrophage oxidative killing mechanisms unless yeast cells are opsonized with antibodies (Eissenberg and Goldman, 1987; Wolf et al., 1987, 1989). Nevertheless, immunoglobulin opsonization of yeast and the ensuing oxidative burst do not affect intracellular growth of *Histoplasma* yeast cells (Howard, 1965). *Histoplasma* yeast cells not only avoid triggering the oxidative burst of murine macrophages but also suppress the production of oxygen radicals by macrophages in response to subsequent stimuli (Wolf et al., 1989, 1992; Ikeda and Little, 1995). Similar to the interactions with murine cells, yeast cells do not stimulate reactive oxygen production from human PMNs unless opsonized with serum components (Schaffner et al., 1986; Schnur and Newman, 1990). On the other hand, unopsonized *Histoplasma* yeast cells can trigger the oxidative burst during the interaction with human macrophages (Bullock and Wright, 1987). In both human macrophages and PMNs, *Histoplasma* yeast cells proliferate despite any production of reactive oxygen compounds (Schaffner et al., 1986; Kurita et al., 1991; Newman et al., 1991). *Histoplasma* yeast survives peroxide challenge in vitro, unlike mycelial-phase cells (Schaffner et al., 1986), but none of the three catalases of *Histoplasma* show yeast phase-specific expression (Johnson et al., 2002).

Intramacrophage Environment

Within the macrophage, *Histoplasma* actively resists killing by lysosomal enzymes. Normal intracellular trafficking of phagosomes involves acidification of the compartment and fusion with lysosomes. *Histoplasma* survival mechanisms may operate at both points in this process. In some experimental systems, *Histoplasma* yeast cells escape destruction by preventing phagosomal fusion with lysosomes (Taylor et al., 1989; Newman et al., 1997, 2006; Strasser et al., 1999). In other assays, *Histoplasma*-containing phagosomes fuse with degradative lysosomes (Eissenberg and Goldman, 1988; Kurita et al., 1991; Newman et al., 1992, 2006), but *Histoplasma* yeast avoids destruction by the acidic hydrolases by maintaining the phagolysosomal pH at 6.0 to 6.5 (Eissenberg and Goldman, 1988; Newman et al., 1992). Modulation of phagolysosomal fusion and/or luminal pH is an active survival strategy expressed by *Histoplasma*, since intracellular compartments containing killed *Histoplasma* yeast become acidified and degrade yeast cells, unlike those with viable *Histoplasma* yeast cells (Newman et al., 1992, 2006; Strasser et al., 1999). *Histoplasma* yeast cells may prevent phagosome acidification in part by reducing the number of vacuolar ATPase proton pumps in the phagosomal membrane (Strasser et al., 1999).

Iron Acquisition

To proliferate within macrophages, *Histoplasma* yeast must acquire iron, which is limited by the host as a means of controlling microbial growth. One of the two iron molecules bound to host transferrin is released at pH 6.0 to 6.5, while the second is released at pH of <5.5. The maintenance of phagosomal pH between 6.0 and 6.4 by *Histoplasma* yeast may represent one strategy to facilitate intracellular survival, because this pH range is acidic enough to liberate one-half of the transferrin-bound iron yet is not low enough to allow activation of acidic hydrolases. Restriction of iron, either by exogenously applied iron chelators or by raising the phagosomal pH, permits macrophages to kill *Histoplasma* yeast, underscoring the critical need to obtain iron within the intracellular environment (Lane et al., 1991; Newman and Bullock, 1994; Newman et al., 1995b). To aid in scavenging limited iron, *Histoplasma* yeast secretes hydroxamate-type siderophores (Burt et al., 1981; Burt, 1982; Howard et al., 2000). These low-molecular-weight iron-chelating compounds can potentially remove iron from host transferrin (Timmerman and Woods, 2001). Reduction of Fe^{3+} to Fe^{2+}, which can cause release of iron from transferrin and siderophores, represents another potential iron acquisition strategy. Three iron reductase activities have been described for *Histoplasma* yeast: (i) a cell surface iron reductase; (ii) a soluble, low-molecular-weight, non-proteinaceous reductant; and (iii) a soluble glutathione-dependent iron reductase (Timmerman and Woods, 1999). In vitro, the glutathione-dependent iron reductase can utilize holotransferrin and hemin as iron sources. Genetic evidence demonstrating the role of these potential iron acquisition mechanisms during intramacrophage growth of *Histoplasma* yeast has only been demonstrated for siderophores (*SID1*) (Hwang et al., 2008), which are discussed below.

Reactive Nitrogen Compounds

With development of a cell-mediated immune response, macrophages become activated to inhibit *Histoplasma* growth. The T-cell-generated cytokine gamma interferon (IFN-γ) triggers this fungistatic state in murine macrophages (Wu-Hsieh and Howard, 1984, 1987) by reducing iron availability (Lane et al., 1991) and/or by stimulating the production of reactive nitrogen compounds (Lane et al., 1994a, 1994b; Nakamura et al., 1994; Brummer and Stevens, 1995; Khemani et al., 1995). On the other hand, human macrophages do not become fungistatic when treated with IFN-γ (Fleischmann et al., 1990), but can become so when incubated with colony-stimulating factors (M-CSF and GM-CSF) and interleukin-3 or by adherence to collagen, which is correlated with increased phagolysosomal fusion (Newman et al., 1997). Production of reactive nitrogen compounds by macrophages only inhibits *Histoplasma* yeast growth (Nakamura et al., 1994; Nittler et al., 2005), suggesting *Histoplasma* can also contend to some degree with

nitric oxide. When *Histoplasma* yeast cells encounter nitric oxide stress, transcription of a number of genes increases, including genes involved in iron acquisition (e.g., *SID1*) and nitric oxide detoxification (Nittler et al., 2005). Overexpression of a P450 nitric oxide reductase (*NOR1*) in *Histoplasma* confers slight resistance to nitric oxide in vitro.

Histoplasma Virulence Factors

Histoplasma virulence is directly linked to the yeast phase. *Histoplasma* cells blocked in the transition from mycelium to yeast are completely unable to cause disease (Medoff, 1986). Furthermore, mycelia are killed by human PMNs, whereas yeast cells remain viable after incubation with these phagocytic cells (Schaffner et al., 1986). These findings lead to the conclusion that the mechanisms underlying *Histoplasma* virulence are specific to the yeast phase. Consequently, this premise forms the basis of numerous studies to identify *Histoplasma* virulence factors (Kumar and Maresca, 1988; Keath et al., 1989; Keath and Abidi, 1994; Colonna-Romano et al., 1998; Hwang et al., 2003). Experimental evidence has validated this rationale, as three of the four defined virulence determinants discussed below are unique to the yeast phase.

CBP

The first virulence factor defined for *Histoplasma* is a calcium binding protein (CBP) that is produced in large amounts by yeast phase cells. This secreted protein exists as a homodimer and binds two molecules of calcium with moderate affinity (K_D [equilibrium dissociation constant], 6 nm) (Batanghari and Goldman, 1997; Beck et al., 2008). The *CBP1* gene, which encodes CBP, is expressed only by yeast phase cells, including during macrophage infection (Batanghari et al., 1998; Patel et al., 1998; Kugler et al., 2000b). Expression of *CBP1* permits growth of *Histoplasma* yeast in vitro when calcium is limited, in contrast to mycelia, which do not express *CBP1* and are inhibited by calcium limitation (Batanghari and Goldman, 1997; Sebghati et al., 2000). Within macrophages, *Histoplasma* produce and release CBP into the lumen of the phagolysosome. By virtue of three disulfide bonds within each CBP monomer, CBP is a highly stable protein, resistant to proteolysis and unfolding (Beck et al., 2008). These properties make CBP highly suited for function within the phagolysosomes during intracellular growth. Genetic deletion of the *CBP1* gene severely impairs the growth of *Histoplasma* yeast within macrophages and greatly attenuates the ability of yeast to colonize the lung (Sebghati et al., 2000). Precisely how CBP promotes the virulence of *Histoplasma* remains unknown. CBP's ability to bind calcium implies that it may function in calcium acquisition within the phagolysosome. Alternatively, structure-based comparisons suggest that CBP may share functions with saposin family proteins that interact with lipids or compete with lysosomal saposin functions necessary for lipid and glycolipid antigen presentation (Beck et al., 2009).

α-Glucan

Entry of *Histoplasma* into phagocytes necessitates an intimate association between host and pathogen. To benefit from this interaction, *Histoplasma* yeast cells conceal immunostimulatory molecules from detection by macrophages. Cell wall β-glucans are characteristic fungal signatures, the detection of which by phagocyte-expressed Dectin-1 receptors triggers antimicrobial defenses and proinflammatory cytokine production (Brown, 2006). Chemotype 2 *Histoplasma* yeast cells (i.e., all but NAm 2 strains) are surrounded by a cell wall layer comprised of α-glucan (Domer, 1971; Kanetsuna et al., 1974; Rappleye et al., 2007). This outer-layer polysaccharide effectively masks the underlying β-glucans from detection by macrophage Dectin-1 (Rappleye et al., 2007). The biosynthesis of the α-glucan virulence determinant involves at least three gene products: α-(1,3)-glucan synthase (AGS1), an α-(1,4)-amylase homolog (AMY1), and UTP-glucose-uridyltransferase (UGP1). Deletion or RNAi-based depletion of any of these results in α-glucan deficiencies and attenuates the virulence of the respective mutant or depleted strains (Rappleye et al., 2004; Marion et al., 2006). While *Histoplasma* α-glucan is primarily composed of α-(1,3)-linked glucan monomers polymerized by the AGS1 gene product, AMY1 is predicted to have specificity for α-(1,4)-linkages, suggesting that *Histoplasma* α-glucan is comprised of at least some α-(1,4)-linked sugars. *UGP1* encodes the uridyltransferase responsible for synthesis of the UDP-glucose monomers. Consistent with its role in pathogenesis, α-glucan is synthesized solely by yeast phase cells; *AGS1* is only expressed by *Histoplasma* in the yeast phase. In addition to *Histoplasma* strains, loss of α-glucan from the cell walls of the other dimorphic fungal pathogens *B. dermatitidis* and *Paracoccidioides brasiliensis* also correlates with attenuated virulence (San-Blas et al., 1977; Klimpel and Goldman, 1987, 1988; Hogan and Klein, 1994). Thus, masking of cells with α-glucan is a general fungal virulence mechanism for escaping host detection.

YPS3

The *YPS3* gene product (yeast phase-specific gene 3) (Keath et al., 1989) is both a secreted and cell wall-associated virulence factor of *Histoplasma* yeast. The YPS3 protein has strong homology to the *B. dermatitidis* adhesin BAD1, except that YPS3 lacks the numerous centrally located invasin-like repeats that have been implicated in BAD1 binding to macrophage complement receptors (Hogan et al., 1995; Newman et al., 1995a). *Histoplasma* yeast cells secrete YPS3, a portion of which becomes associated with the yeast cell wall through interaction with chitin (Weaver et al., 1996; Bohse and Woods, 2005). The C-terminal region of BAD1 mediates binding to chitin and the homologous region in YPS3 likely functions in chitin binding as well (Brandhorst et al., 2003). Strains from each of the *Histoplasma* phylogenetic groups possess the *YPS3* gene, with a polymorphic variable-length repeat in the central region of YPS3 correlating with the phylogenetic groupings (Bohse and Woods, 2007a). However, production of YPS3 is confined to NAm 2 strains at the level of transcription (Keath et al., 1989; Bohse and Woods, 2007a). The *YPS3* promoter shares some sequence elements with the BAD1 upstream region, and both promoters support transcription in the heterologous host (Rooney et al., 2001; Rooney and Klein, 2004). Decreasing YPS3 production in G217B (NAm 2) does not affect macrophage parasitization in culture but does attenuate *Histoplasma* virulence in vivo, particularly the ability of yeast cells to disseminate to internal organs (Bohse and Woods, 2007b). The expression of YPS3 in NAm 2 strains may thus explain the greater fungal burdens in spleens observed following G217B respiratory infection compared to infections with Panamanian class strains of *Histoplasma* (Mayfield and Rine, 2007).

Siderophores

Maximal virulence of *Histoplasma* depends on efficient acquisition of iron, which is facilitated by siderophore production. Growth of yeast cells under conditions of iron limitation induces transcription of seven genes with roles in siderophore production (based on sequence homologies [Hwang et al., 2008]). Six of these genes (*SID1*, *SID3*, *SID4*,

NPS1, *OXR1*, and *ABC1*) cluster within a single genomic region, and the promoters of each iron-regulated gene contain a potential *cis*-regulatory element. The *SID1* gene encodes L-ornithine monooxygenase, the enzyme that catalyzes the first committed step in siderophore production. Deletion of *SID1* impairs the ability of *Histoplasma* yeast to replicate within cultured macrophages, confirming the role of siderophores in intracellular growth. In animal models, loss of siderophore biosynthesis does not significantly affect lung colonization, possibly due to redundant mechanisms for iron acquisition (e.g., iron reductase activities). Nevertheless, *sid1*Δ mutant yeast cells are outcompeted by *SID1(+)* yeast during murine respiratory infection, particularly at day 15 postinfection, when maximal IFN-γ production is predicted to decrease intracellular iron availability. It is unknown whether siderophore biosynthesis also affects mycelial-phase growth of *Histoplasma*.

SUMMARY

The molecular details underlying the mechanisms promoting *Histoplasma* virulence are now beginning to be revealed with the application of molecular genetics (Fig. 3). These studies demonstrate that the result of the interaction between *Histoplasma* yeast and the macrophage is influenced by yeast cell surface components that dictate which host receptors are engaged; the HSP60 adhesin facilitates binding to macrophages, while the α-glucan polysaccharide masks additional detection. Once within the macrophage, *Histoplasma* yeast cells modify their intracellular compartment to prevent destruction by lysosomal enzymes; however, the virulence factors mediating these steps remain largely undefined. *Histoplasma* yeast cells secrete CBP and iron-scavenging siderophores to facilitate intracellular survival and replication, which ultimately leads to the demise of the macrophage and spread of the fungal burden.

The completion of genome sequences for three phylogenetically distinct strains and the development of transcriptional microarrays is launching genomics-based studies of *Histoplasma*. Gene expression analyses, as exemplified by the identification of *SID1*, can be used to gain molecular footholds for probing some of the outstanding issues in *Histoplasma* biology, such as which genes control phase transition processes, which genes are important for intracellular growth, or how *Histoplasma* modifies and maintains its intracellular environment. YPS3 highlights the issue of mechanistic differences among *Histoplasma* strains, the study of which will be particularly aided by bioinformatic analyses and genome comparisons. Investigation of geographically overlapping yet nonrecombining species at the genome level should also provide an important perspective on *Histoplasma* evolution. Genome comparisons with other species may also enhance our understanding of the issue of dimorphism.

The virulence factors identified for *Histoplasma* have all relied upon the development of reverse genetics methodology to functionally demonstrate the role of suspected genes in virulence. As genomics-based studies identify additional candidate genes for virulence and other aspects of *Histoplasma* biology, these same reverse genetics tools will prove essential. The ability of forward genetics to identify novel or unsuspected genes involved in the biology of *Histoplasma* is now possible through insertional mutagenesis techniques. This approach has succeeded in identifying two components, DRK1 and RYP1, that govern adoption of the yeast phase, although their connections to the biochemical and morphological features of the conversion process remain to be elucidated. These complementary approaches, the ability to remove a gene of interest or to identify a gene based on mutant phenotypes, will advance our molecular understanding of this clinically important and biologically fascinating fungal pathogen.

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The Fungal Pathogen *Candida albicans*

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For the readers of a book on filamentous fungi, the mention of *Candida albicans* may seem like an odd interjection. *C. albicans* is pleiomorphic, with the ability to grow as yeast cells (also called blastospores), pseudohyphae, true hyphae, or a mixture of those cell types (Sudbery et al., 2004; Whiteway and Bachewich, 2007). Yeast-like cells come in two flavors, "white" and "opaque" (Soll, 2004). An additional cell type, the chlamydospore, is produced under special environmental conditions but is not self-propagating (Whiteway and Bachewich, 2007). Thus, *C. albicans* is a facultative filamentous fungus. In addition, *C. albicans* lacks the elegant genetic features that attracted Beadle and Tatum to *Neurospora* (Raju, 1999) and have been an underpinning of the study of fungi ever since. The sexual behavior of *C. albicans*, as we now know it, is a patchwork of a vestigial mating system combined with what seems like panic-induced chromosome loss (Noble and Johnson, 2007). Admittedly, recent findings implicating a meiotic gene ortholog in chromosome loss may be the first flicker of hope for a complete sexual cycle (Forche et al., 2008), but even so, we would have to say that *C. albicans* is among the most modest of sexually active fungi. To add insult to injury, the organism is naturally diploid (Noble and Johnson, 2007), so that a null mutant phenotype is most apparent only after successive disruption of two alleles at a locus of interest. And concerns about secondary mutations and deviations from euploidy (Selmecki et al., 2005) require additional genetic manipulations, generally complementation of the defined mutation, before the function of the affected gene can be asserted with confidence (Noble and Johnson, 2007). It will come as no surprise that the phrase "*Candida* genetics" was long considered to be an oxymoron, and presentations on the subject at meetings were often relegated to the morning session after the banquet. What possible reason could inspire a scientific community to study *Candida* species other than distaste for after-banquet evening activities? Could it be the unfortunate fate of those who fail to learn the steps of meiosis in college?

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Candida species are the major fungal pathogens of humans, with the ability to cause superficial, mucosal, or deep tissue infections. They are ranked fourth among nosocomial pathogens, with an associated mortality rate of 40% (Spellberg et al., 2006; Pfaller and Diekema, 2007). They cause vaginitis in approximately 40% of total humans (Fidel and Sobel, 1996). They cause oral or esophageal infections in virtually all AIDS patients who lack access to highly active antiretroviral therapy (de Repentigny et al., 2004). Available antifungal therapies are limited (Spellberg et al., 2006). Thus the study of *Candida* species is driven by the need to understand infection and pathogenesis, to diagnose infection early enough so that existing therapies may be effective, and to develop new therapies based upon our understanding of the organisms' basic molecular and cell biology. There is also the emerging concept that virulence traits might be targeted for therapeutics as well (Cegelski et al., 2008), with some encouraging success in that direction (Ibrahim et al., 2006). Therefore, medical need is the major driving force behind the study of pathogenic *Candida* species. Because *C. albicans* is the species most frequently isolated from patients, we focus on that species in particular.

Research on *C. albicans* is driven by the major questions regarding any infectious microbe. How is infection initiated? What functions promote pathogenesis? How does drug resistance arise, and how is it transmitted? How does the organism adapt to diverse niches? What microbial processes are useful therapeutic targets? We address these questions with a toolbox that includes genetics, cell biology, and molecular analyses such as expression profiling and proteomics (Berman and Sudbery, 2002; Nantel et al., 2002; Alvarez and Konopka, 2007; Noble and Johnson, 2007). Many of these tools derive from, or have been augmented by, the genome sequence (Jones et al., 2004; Braun et al., 2005) and its availability online in many user-friendly formats (Arnaud et al., 2007; Rossignol et al., 2008). In the course of addressing these questions, the field has been led into many areas of more basic biology, including the nature of *C. albicans* sexual processes, the nature of numerous cell biological processes, the evolutionary relationships among fungal species, the competitive relationships between fungal and bacterial microflora, how retooling of the genetic code may have arisen, and the nature of epigenetic switches

between novel cell types. Ultimately, we think of each study as a piece of the infection puzzle, a step toward assembling the big picture, and an opportunity to reduce risk or accelerate recovery.

In this chapter we introduce first the medical problem and then animal models of infection and genetic tools for *C. albicans* manipulation. The field of basic *C. albicans* research is too broad to cover all areas of inquiry at this time. Thus, we finish the chapter with focus on three topics—morphogenesis, adherence, and azole drug resistance—that have long held the attention of our research community, in order to give the reader a sense of key questions and prospects for future study.

EPIDEMIOLOGY

C. albicans and many other *Candida* species are colonizers of the human gastrointestinal (GI) and genitourinary tracts (Fidel and Sobel, 1996; Donskey, 2004). They seem to be benign most of the time and are thus considered commensal organisms. However, their intimate relationship with us provides a staging area from which they mount an infection when the opportunity arises. Such an opportunity is presented by diabetes, by AIDS, by extremes of youth or age, by the presence of an indwelling catheter or other implanted device, by the elimination of competitors with broad-spectrum antibiotics, and by many other risk factors that have become familiar features of our existence (de Repentigny et al., 2004; Spellberg et al., 2006; Pfaller and Diekema, 2007). Purists have argued on occasion that virulence can only be defined for primary pathogens, those that infect healthy individuals. However, realists have revised that perspective with the understanding that there is a vast range of immune function among "healthy" people (Casadevall and Pirofski, 2003). Infection reflects the delicate balance of power between host and microbe.

Candida species are the fourth most common cause of nosocomial bloodstream infections in the United States, with an incidence rate of 8 cases per 100,000 in the general population. The attributed mortality rate is almost 50% for bloodstream and disseminated infections, and a higher frequency is acknowledged among neonates and African-Americans (Gudlaugsson et al., 2003; and see http://www.cdc.gov/nczved/dfbmd/disease_listing/candidiasis_ti.html). One recent study estimates infection rates among neonates at 466 infections per 100,000 and a fourfold-higher rate among African-American neonates (Pfaller and Diekema, 2007). Neonates have several risk factors for infection, including a suppressed (underdeveloped) immune system, medical implants, and use of broad-spectrum antibiotics, which helps to explain their high rate of infection.

Over 17 different *Candida* species cause invasive candidiasis in humans; 5 of these account for more than 90% of invasive infections (Pfaller and Diekema, 2007). In the United States, *C. albicans* is the most common, followed by *C. glabrata* and then by *C. tropicalis* and *C. parapsilosis*; other species occur much less frequently (Kauffman, 2006). Gudlaugsson et al. found *C. albicans* to be responsible for 68% of cases of patients with *Candida* bloodstream infections (Gudlaugsson et al., 2003). *C. glabrata* and *C. parapsilosis* followed at 17 and 13%, respectively, while *C. tropicalis*, *C. lusitaniae*, and *C. krusei* were each responsible for 10% or less of such infections (Gudlaugsson et al., 2003). Crude mortality was not significantly different between *Candida* species. For example, the crude mortality rates for *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *Candida tropicalis* are 62, 50, 46, and 70%, respectively (Gudlaugsson et al., 2003).

The relative frequencies of infecting *Candida* species are influenced by geography and demographics. For example, in some countries, *C. parapsilosis* and *C. tropicalis*, not *C. glabrata*, are the more frequently causative *Candida* species in patients with bloodstream infections (Pfaller and Diekema, 2007). In addition, *C. parapsilosis* tends to be associated with infections in infants and neonates, whereas *C. tropicalis* affects neutropenic patients and those with hematologic malignancies (Pfaller and Diekema, 2007). Thus, several *Candida* species are highly significant medically, with a relative importance that depends on the context of infection and the particular associated risk factors.

INFECTION MODELS

With the *C. albicans* research community's motivation to understand disease comes a broad but unifying question: how does a phenomenon that we observe in vitro pertain to the host-pathogen interaction in vivo? There have been some studies in which patient samples are used directly to address such questions (see Naglik et al., 1999, for example), but far more often we employ an infection or colonization model. Most studies of virulence have been accomplished with mouse models of disseminated infection (see Sanchez et al., 2004, for example), mucosal infection (Samaranayake and Samaranayake, 2001), or GI tract colonization and dissemination (Clemons et al., 2006; Koh et al., 2008). However, there is also a piglet model of GI tract colonization and transit into the bloodstream (see White et al., 2007), venous catheter biofilm formation with rats or rabbits (Andes et al., 2004; Schinabeck et al., 2004), and ex vivo models that include infection of reconstituted human oral or vaginal epithelium (Schaller et al., 1998, 2005). A whole-blood model has been combined with fractionation to define the major host cell types that evoke *C. albicans* responses in that context (Fradin et al., 2003). These models have all proven to be useful for recapitulation of certain types of disease or aspects of pathogenesis.

There are also host cell interaction models, which permit more detailed analysis of aspects of disease. The source of host cells can be human, thus overcoming the species limitation of other infection models. The endothelial cell interaction model has been used to focus on mechanisms that permit tissue invasion from the bloodstream (Sanchez et al., 2004), because endothelial cells form the lining of blood vessels. Epithelial cell interaction models have been used to elucidate aspects of mucosal infection (Staab et al., 1999). And interactions of *C. albicans* with innate immune cells, including macrophages and neutrophils, have been used to understand host defenses as well as to probe mechanisms of *C. albicans* growth and survival following endocytosis (Lorenz et al., 2004; Netea et al., 2006; Rubin-Bejerano et al., 2007). These host cell interaction models offer the opportunity to study both host and pathogen at high resolution.

There is a growing selection of minihost models. Here we use infection of *Drosophila melanogaster*, *Caenorhabditis elegans*, or *Galleria mellonella* (Mylonakis and Aballay, 2005; Chamilos et al., 2006; Fuchs and Mylonakis, 2006). These models cannot recapitulate all of the complexity of mammalian infection, but they lend themselves to high-throughput screens that would be prohibitive for many reasons with mammalian hosts.

With such an array of infection models available, how can one choose among them? One sort of criterion is how well the model reflects the specific aspect of infection under study. For example, infections involving biofilms or

surface-associated C. *albicans* communities may be reflected substantially with catheter infection models, mucosal infection models, or even reconstituted epithelium infection models. A second criterion is the level of resolution required. For example, elucidation of a host surface receptor that mediates C. *albicans* endocytosis and the cognate C. *albicans* surface ligand derived from studies with human endothelial cells (Phan et al., 2007). On the other hand, the relationship between filamentation and growth during infection was addressed with a disseminated infection model (Saville et al., 2003). The connection between GI tract colonization and allergic responses was of course made with an animal model (Noverr et al., 2005). Hence, no one infection model is perfect for all questions; rather, we match the model to the question.

The choice of infection model is indeed critical in defining traits and genes required for virulence. This point is illustrated by recent studies that assess virulence of mutant strains in murine infection models. For example, the protein kinases Cka2 and Tpk2 are required for oral infection but not disseminated infection (Park et al., 2005; Chiang et al., 2007). On the other hand, the phosphatidylinositol 4,5-bisphosphate regulator Irs4 is required for disseminated infection but not oral infection (Badrane et al., 2005). Finally, the transcription factor Efg1 is required for virulence in both oral and disseminated infection models (Lo et al., 1997; Park et al., 2005). It perhaps makes intuitive sense that the demands of infection would differ in each context. In a practical sense, the nature of the infection model can affect our appraisal of the significance of a gene in the infection process.

Just as infection models use an array of different hosts or tissues, they sometimes employ fungi that are seldom isolated as pathogens to address specific questions. One of the earliest examples is the use of *Saccharomyces cerevisiae* as a vector for heterologous expression. Such studies have shown that surface proteins Als1, Als3, and Eap1 mediate binding of C. *albicans* to host cells (Fu et al., 1998b; Li and Palecek, 2003; Sheppard et al., 2004). There is always the concern that the alternate C. *albicans* genetic code—the CUG codon generally specifies serine rather than the universal code's leucine (Gomes et al., 2007)—may impair function in heterologous expression experiments, but a potential positive result is incentive enough to overcome some experimentalists' trepidation. A second kind of study defined nutrient availability in vivo in a mouse disseminated infection model through growth assays with *S. cerevisiae* mutants (Kingsbury et al., 2006). Among filamentous fungi, *Aspergillus nidulans* has had promising application in an animal infection model (Bignell et al., 2005). So we often look toward experimentally accessible animals as models for humans and manipulatable nonpathogenic fungi as models for pathogens in order to guide our thinking about disease.

GENETIC AND GENOMIC TOOLS

Thus far we have discussed the nature of human disease and how an array of systems permit it to be modeled. How can we derive mechanistic information about the pathogen and how it interacts with the host? Classical genetic approaches provided some basic insight into C. *albicans* biology and pathogenesis (Molero et al., 1998), but the development of molecular tools and genomic sequence information has driven a surge of mechanistic studies over the past decade.

The genomic sequence of clinical isolate SC5314 was published in 2004 (Jones et al., 2004), followed by a community annotation in 2005 (Braun et al., 2005), but both sorts of information had been made available several years previously. C. *albicans* comprises 6,354 genes and has a genome size of approximately 14.8 Mb (Braun et al., 2005). The latest figure from the Candida Genome Database gives a chromosome length of approximately 14.3 Mb (as of 6 April 2009). C. *albicans* is a diploid organism divided between eight chromosome pairs, which are designated numbers 1 through 7 (from the largest, ~3 kb, to the smallest, ~1 kb) and R (ribosomal DNA, ~2.5 kb) (Jones et al., 2004). The gene set reveals an extensive range of catabolic functions (Braun et al., 2005), as well as large gene groupings encoding proteases, lipases, and cell wall proteins that are not found in such sizeable families in *S. cerevisiae* (Berman and Sudbery, 2002). Finally, it is noteworthy that C. *albicans* is highly heterozygous, with one polymorphism in 237 bases in strain SC5314 (Jones et al., 2004) and about one-half that many in strain WO-1 (Butler et al., 2009). Heterozygosity for recessive mutations permits rapid acquisition of new traits, such as drug resistance (Dodgson et al., 2004), through mitotic recombination.

The sequencing project was initiated at the Stanford Genome Technology Center, where whole-genome shotgun sequencing was undertaken with strain SC5314. Since then, the sequences of C. *albicans* strain WO-1, C. *tropicalis*, C. *guilliermondii*, and C. *lusitaniae* have been published (Butler et al., 2009). It is now feasible to compare sequences among *Candida* species, contrast with *S. cerevisiae*, and compare to other genomes to discover fungus-specific genes. Genes encoding novel products (particularly those not seen in *S. cerevisiae*), those only seen in other fungi, or those that are completely novel are now research focal targets. This research is central to development of new drugs based on new drug targets as well as for expansion and improvement of tools for more rapid diagnoses.

Transformation

How can we get DNA into our organism? C. *albicans* was first transformed through use of a spheroplasting protocol (Kurtz et al., 1986). Such protocols are generally considered to be fingernails on a chalkboard to the uninitiated, but in fact C. *albicans* spheroplasts are so hardy that the procedure is relatively quick and painless. The most popular method today is a LiOAc transformation protocol developed by Braun and Johnson (Braun and Johnson, 1997) and based on protocols for *S. cerevisiae*. It may be useful to keep in mind that a commercial LiOAc kit, the Frozen EZ-II kit (www.zymoresearch.com), works quite well. Electroporation can also be used (see Schaub et al., 2006). Thus, there is no good excuse for being unable to transform C. *albicans*.

Markers

A number of genetic markers are routinely used to select for DNA uptake and integration. The molecular genetic era was ushered in through Myra Kurtz's development of a reliable transformation protocol that yielded integrative transformants (Kurtz et al., 1986). Systematic reverse genetics was then enabled through Fonzi and Irwin's heroic creation of the homozygous *ura3/ura3* deletion strain CAI4 (Fonzi and Irwin, 1993). They chose to develop the *URA3* marker because Alani and Kleckner had worked out an extremely clever marker cassette, the "URA blaster," that employed recombination between flanking direct repeats to loop out the intervening *URA3* marker (Alani et al., 1987). Thus, the URA blaster permits recycling of the *URA3* marker, enabling its repeated use. The need to disrupt two alleles of any gene of interest to create a null mutant could be satisfied with a single marker. Fonzi and Irwin also adapted the URA blaster for use in C. *albicans* (Fonzi and Irwin, 1993).

The key advantage of *URA3* that supported use of the URA blaster was that it could be selected for Ura^+ as well as against it, through selection for resistance to 5-fluoroorotic acid (5-FOA^R). However, it was quickly realized that *ura3/ura3* mutants are attenuated in a mouse disseminated infection model and thus, that mutant and complemented control strains all had to be Ura^+ for an informative comparison. This was conveniently achieved by retaining the intact URA blaster in the second disrupted allele for the homozygous mutant and then introducing a wild-type allele for complementation on a *URA3*-marked DNA construct into the Ura^- homozygous mutant. This approach is perfectly suitable to permit Ura^+ growth of both strains in vitro, but a potential concern is that the *URA3* gene in the mutant has a different flanking sequence context from that in the complemented strain; the flanking sequence might modify *URA3* expression levels. An early indication that this was the case came from direct enzyme assays conducted in the Becker lab (Lay et al., 1998). Subsequent studies from the groups of Fonzi and Sundstrom revealed the biological impact of this situation: attenuation of *hwp1/hwp1* mutant in a disseminated infection model was at least partially a consequence of its *URA3* expression level (Sundstrom et al., 2002; Sharkey et al., 2005). A solution to this problem, which permits use of *URA3*, has been to maintain the marker in the same sequence context in both mutant and complemented strains, by virtue of integration at a neutral locus (Brand et al., 2004), or by maintaining the *URA3*-marked mutant allele in both the mutant and complemented strains through use of multiple gene disruption markers (Wilson et al., 1999). Other terrific solutions avoid the *URA3* marker entirely, either by using a dominant drug resistance marker (*NAT1* or *SAT1* cassettes, which confer nourseothricin resistance [Reuss et al., 2004; Shen et al., 2005]) or by using a set of markers that do not affect virulence in the disseminated infection model (Noble and Johnson, 2005). Among these, the use of dominant drug resistance markers seems the most generally useful, because it would be virtually impossible to test every auxotrophic marker for impact in every infection model. The limitation in implementing drug resistance selection has been that *C. albicans* is naturally resistant to most available antimicrobials (discussed by De Backer et al., 2000). As we will see, though, the development of a recyclable drug resistance marker, the *SAT1* flipper, has circumvented that limitation.

The URA Blaster and URA Flipper

The URA blaster technique permits orderly disruption of both alleles of one or several *C. albicans* genes. The cassette (Fonzi and Irwin, 1993) comprises the *URA3* gene flanked by direct repeats of a functionally inert sequence, the *Salmonella his*G gene. Transformants that carry the cassette are selected through their Ura^+ phenotype. Recombination between the *his*G sequences can then occur spontaneously, resulting in loss of *URA3*. These Ura^- recombinants are selected by virtue of their resistance to 5-FOA. The resulting Ura^- strain can then be used as a recipient for another Ura^+ transformation, and subsequent repetition of the 5-FOA selection yields a Ura^- strain again.

The *his*G repeats are a bit too large (1.1 kbp) to permit routine PCR amplification of the entire URA blaster cassette. This problem is circumvented by the URA blaster, or *URA3-dpl200*, which has only 200-bp flanking direct repeats (Wilson et al., 2000).

The requisite 5-FOA selection presents two significant problems. First, 5-FOA treatment can cause chromosome aberrations in Ura^+ survivors (Wellington and Rustchenko, 2005). Second, there are two simple ways that a heterozygous *YFG1/yfg1::hisG-URA3-hisG* strain can become Ura^- and 5-FOA^R: it can go through direct-repeat recombination to "pop out" the *URA3* gene, yielding the desired *YFG1/yfg1::hisG* heterozygote, or it can undergo mitotic recombination between homologous chromosomes, yielding the dreaded *YFG1/YFG1* homozygote. In view of these problems, Morschhauser and colleagues devised the URA flipper cassette (Morschhauser et al., 1999). This cassette includes the *URA3* gene along with the *S. cerevisiae FLP* gene, which specifies a site-specific recombinase. The *FLP* coding region is fused to the *C. albicans SAP2* promoter, permitting regulated expression of the recombinase (off in typical yeast extract-peptone-dextrose or synthetic defined growth media, on in bovine serum albumin-containing medium). Finally, the entire cassette is flanked by 34-bp *FRT* target sites for Flp. Thus, cassette integration into the genome can be demanded by selecting Ura^+ transformants; excision of the cassette occurs at high frequency in bovine serum albumin medium to yield Ura^- segregants with a 34-bp *FRT* site at the site of integration. The excision frequency is high enough (~20% of cells) that there is no need for 5-FOA selection (Morschhauser et al., 1999).

Dual-Marker Systems

The use of strains with two auxotrophic markers permits the creation of homozygous insertion or deletion mutants through two successive transformations, first selecting for one marker and then for the second. This strategy was first employed by Kurtz and Marrinan to create a *hem3::URA3/hem3::LEU2* homozygous insertion mutant (Kurtz and Marrinan, 1989). Strains now commonly used for this approach include BWP17 (*ura3/ura3 his1/his1 arg4/arg4* [Wilson et al., 1999]) and SN152 (*his1/his1 leu2/leu2 arg4/arg4* [Noble and Johnson, 2005]). Improved marker cassettes for both strains are available (Gola et al., 2003; Schaub et al., 2006). Strain SN152 has the added advantage that its markers do not affect virulence in the murine disseminated infection model (Noble and Johnson, 2005). Each of these commonly used strains has three markers: two markers for successive disruption of each allele of the gene of interest and a third one to permit complementation of the mutation with a wild-type copy of the gene. The complementation step, which fulfills Koch's molecular postulates (Falkow, 2004), provides clear evidence that the defined mutation is the cause of the observed phenotype.

A useful hybrid between dual-marker systems and the URA blaster is the split-marker cassette *UAU1*, which permits selection for homozygous mutants after a single transformation (Enloe et al., 2000). The selectable marker in *UAU1* is *ARG4*. That *ARG4* marker is flanked by direct repeats that are in one way similar to the *his*G repeats in the *URA* blaster: they enable excision of the *ARG4* marker through homologous recombination. But the repeats in *UAU1* have a unique property as well: each is a nonfunctional fragment of *URA3*, and recombination stitches together an intact *URA3* gene. Hence, the cassette can exist in two states, an Arg^+ Ura^- state and an Arg^- Ura^+ state. This property enables selection for homozygous mutants as follows: an initial heterozygous Arg^+ transformant (*yfg1::UAU1/YFG1*) is grown overnight, and occasional mitotic recombination or gene conversion events yield homozygous segregants in the population (*yfg1::UAU1/yfg1::UAU1*). Those homozygotes can undergo recombination within one of the *UAU1* cassettes to yield an intact *URA3* gene (*yfg1::UAU1/yfg1::URA3*). Such segregants may be selected through their unique Arg^+ Ura^+ phenotype. One might expect the segregants to be rare, but

there are typically 10 to 30 Arg^+ Ura^+ segregants in 100 μl of a yeast extract-peptone-dextrose overnight culture. Roughly one half of such segregants are homozygous mutants; the other half have more complex structures and retain a wild-type copy of *YFG1* (Enloe et al., 2000). Of course, for *UAU1* insertions in essential genes, no homozygotes are recovered. The fact that a single *UAU1*-containing DNA construct can be used to make a homozygous mutant has permitted insertional mutagenesis on a reasonably large scale (Davis et al., 2002; Nobile et al., 2003; Bruno and Mitchell, 2005; Nobile and Mitchell, 2005; Richard et al., 2005; Bruno et al., 2006; Nobile and Mitchell, 2009). This advantage is offset by the concern that the homozygous mutants generated by recombination may be homozygous for linked mutations that may affect phenotype. The concern has been addressed by complementation, analysis of several independent mutants, and follow-up construction of a "clean" mutant by traditional methods.

SAT1 Flipper

The basic strategy and structure of the *URA flipper* has been adapted for use with drug resistance markers, permitting targeted gene disruption in otherwise wild-type strains. The original inception, the MPAR flipper (Morschhauser et al., 2005), was workable but employed the relatively noisy selection for mycophenolic acid resistance. The similarly developed *SAT1* flipper (Reuss et al., 2004) utilizes a cleaner selection for nourseothricin resistance. The advantages of the system include isolation of transformants within 1 day as well as the high specificity with which integration into the target locus takes place; targeting can be directed by fairly long flanking regions that are easily cloned into the *SAT1* flipper vector.

Heterologous Expression

Much early gene discovery with *C. albicans* relied upon heterologous expression of *C. albicans* genes, primarily in *S. cerevisiae*. This strategy was useful for cloning of nutritional markers (see Kurtz et al., 1986), drug resistance determinants (see Ben-Yaacov et al., 1994), and adherence determinants (see Fu et al., 1998a, 1998b; and Li et al., 2007). The approach has remained useful for analysis of *C. albicans* cell surface adhesins, because *S. cerevisiae* has little native adherence ability to many biomedically relevant substrates (Rauceo et al., 2004; Sheppard et al., 2004; Li and Palecek, 2008).

The greatest general limitation to the approach is that the CUG codon specifies a serine residue in *C. albicans*, not a leucine residue as in *S. cerevisiae* and most other organisms (Gomes et al., 2007). Although CUG codon usage is lower in *C. albicans* than in *S. cerevisiae* (Butler et al., 2009), there is the potential for mistranslation of over 30% of *C. albicans* open reading frames in *S. cerevisiae*. It may be useful for the future to consider heterologous expression of *C. albicans* genes in a nonpathogenic species that shares the unusual translation rules (Butler et al., 2009), such as *Debaryomyces hansenii* (Dujon et al., 2004).

Fluorescent and Reporter Protein Fusions

Promoter green fluorescent protein (GFP) and protein-GFP fusions have been invaluable for examining protein localization (for an example, see Bauer and Wendland, 2007; and Alvarez et al., 2008) and targeting sequences (see Mao et al., 2008), marking cell subpopulations (for an example, see Nobile and Mitchell, 2005), and defining gene regulation in vitro and in vivo during infection (see Enjalbert et al., 2007). Codon-optimized GFP-coding regions were initially described by Cormack et al. (Cormack et al., 1997) and Morschhauser et al. (Morschhauser et al., 1998). A widely used set of *C. albicans* GFP, cyan fluorescent protein, and yellow fluorescent protein fusion vectors have been developed by Gale and colleagues (Gerami-Nejad et al., 2001). Gola et al. have also described a vector for GFP fusion creation (Gola et al., 2003). A recently developed construct facilitates internal GFP fusions (Gerami-Nejad et al., 2009). Finally, the Ernst group has developed a flavin mononucleotide-based fluorescent protein reporter that can be used in hypoxic environments (Tielker et al., 2009).

C. albicans does not express native β-galactosidase activity, a prospective asset that was initially not exploited because the *Escherichia coli lacZ* gene is nonfunctional in *C. albicans*. Other β-galactosidase genes have been developed for use in *C. albicans*, including *Kluyveromyces lactis LAC4* (Leuker et al., 1992) and *Streptococcus thermophilus lacZ* (Uhl and Johnson, 2001). A user-friendly *S. thermophilus lacZ* cassette that simplifies fusion gene construction has been described recently (Lavoie et al., 2008).

The topic of reporter genes has been reviewed recently with a very useful methodological focus by Sturtevant (2009).

Promoter Fusions

Expression analysis is fundamental to characterization of gene function. Alterations to endogenous expression are required for this, which necessitates a high-quality varied promoter collection. Berman and Sudbery (2002) list *ADH1*, *ACT1*, *GAL1*, *PCK1*, *MAL2*, and *MET3* among the currently available selection. User-friendly cassettes for promoter exchange have been developed by several groups (Gola et al., 2003; Gerami-Nejad et al., 2004). Our lab has made use of the *TEF1* and *TDH3* promoters to overexpress transcription factor target genes, permitting phenotypic rescue of mutants in many cases (Nobile et al., 2006a, 2008b). We believe that this relatively simple overexpression-rescue approach will be extremely useful for making gene function connections and defining regulatory networks.

Perhaps the most broadly useful regulatory rewiring approaches involve the use of *tet* repressor derivatives that can either repress (Nakayama et al., 2000) or activate (Park and Morschhauser, 2005) gene expression in the presence of tetracycline or doxycyline. The key advantage of these systems is that gene expression can be manipulated in animal infection models, thus permitting critical analysis of virulence gene function during infection (Saville et al., 2003). Perhaps the most general concern with any regulated expression system is that the repressed state of a gene—when it is turned off—may yield a basal level of expression that has biological impact. Some clear examples are presented by Roemer and colleagues (Roemer et al., 2003), who nonetheless were able to use a *tet*-based system to screen among 823 potentially essential *C. albicans* genes. However, the basal expression problem has been most clearly addressed through development of a very clever doxycyline-inducible gene deletion system (Park and Morschhauser, 2005). Although it may take a few generations to deplete preexisting gene product after deletion of the gene, at least we can be confident that there is no new expression once the gene is deleted.

Databases and Information Sources

Among valuable databases that are critical to postgenomic era research are these.

- *Candida* Genome Database: www.candidagenome.org
- Pasteur Institute Database: http://genodb.pasteur.fr/cgi-bin/WebObjects/CandidaDB

- The Conway Institute (for bioinformatics analyses): http://actin.ucd.ie
- *Saccharomyces* Genome Database: www.yeastgenome.org
- The Biotechnology Research Institute: http://candida.bri.nrc.ca/candida/index.cfm
- The Fungal Genetics Stock Center: http://www.fgsc.net/candida/FGSCcandidaresources.htm

SIGNALING IN MORPHOGENESIS AND PATHOGENESIS

There are two primary vegetative forms of proliferation for *C. albicans*: yeast and hyphae. These differ not only in morphology but also in cell cycle (Berman, 2006). The *yeast form* (also referred to as a blastospore) entails cells elongating and budding off the mother cell into the daughter cell. Chromosomes are separated between mother and daughter by spindle pole bodies (Whiteway and Bachewich, 2007). Yeast cells are mainly round or ovoid and separate easily from each other (Berman and Sudbery, 2002). The *hyphal form* involves constant growth at the tip and yields an elongated tube. Cells separate by septa. In hyphal growth, both a polarisome and a Spitzenkörper are present at the tip of the hypha. Nuclear division occurs in the germ tube, and one nucleus moves back into the mother cell and the second moves into the elongating tubes (Whiteway and Bachewich, 2007).

The hyphal form is subdivided into true and pseudohyphal types. *Pseudohyphae* appear as chains of elongated blastospores joined end to end and tend to have an ellipsoid profile. Growth usually is in a branching configuration and is suggested to assist in seeking nutrients away from the parent cell (Berman and Sudbery, 2002). The pseudohyphal form often coexists with yeast and hyphal forms in vegetative cultures and during infections. *True hyphae* appear as filaments without constrictions at the cell-cell junctions. The cells are long and highly polarized (Berman and Sudbery, 2002).

There are important differences between the three states (yeast, pseudohyphal, and true hyphal). These include (i) extent of polarized growth (the degree of polarity is more pronounced in the hyphal form); (ii) septin ring position and true septum with regard to the mother cell; (iii) nucleus movement with regard to the mother cell; and (iv) separation of the daughter cell (Berman and Sudbery, 2002).

Other distinct morphologies of *C. albicans* include (i) opaque form (usually mating-competent cells) and (ii) the chlamydospore (usually forms in suboptimal environmental conditions). Hyperextended buds can also form, usually as a result of cell cycle-related stresses (Whiteway and Bachewich, 2007).

Opaque form cells elongate mating projections (shmoo) in response to mating pheromone. The nucleus moves into the mating projection and following fusion to an opposite mating type undergoes fusion with another nucleus (Whiteway and Bachewich, 2007).

Chlamydospores are formed at the end of suspensor cells. They are larger than yeast form cells and have a thicker cell wall. Nuclear division occurs within the suspensor cell, followed by moving of the daughter nucleus to the chlamydospore across a septin. Chlamydospores are often noted for their elaborate "septin-derived substructure" (Whiteway and Bachewich, 2007).

Relationship between Morphogenesis and Virulence

No discussion of *C. albicans* virulence can begin (Cutler, 1991) without consideration of hyphal formation as a virulence trait. Why would anyone think that a morphogenesis program may be tied to virulence? Numerous intriguing connections have been known for some time. First, *C. albicans* hyphae are readily apparent in infected tissue. Second, *C. albicans* is induced to produce hyphae by incubation conditions that mimic the mammalian host environment, i.e., the presence of serum at 37°C. The more recent discovery that 5% CO_2 also induces hyphal formation (Klengel et al., 2005) is a further connection to growth in vivo. Third, it makes intuitive sense that a fairly rigid rod-like form would have the physical capacity to invade tissue, though this is a point we will revisit. And finally, the production of hyphae is accompanied by a substantial change in the spectrum of surface and secreted proteins (for an example, see Sohn et al., 2003), so that hyphae are more adherent than yeast cells in numerous assays and also secrete high levels of hydrolases such as the Sap4, Sap5, and Sap6 aspartyl proteases (Naglik et al., 2003). Adherence and hydrolase production are known to promote pathogenesis in diverse infections with an array of organisms (Finlay and Falkow, 1997); thus, the overall hyphal developmental program seems to be coupled to virulence factor elaboration.

The first compelling evidence that hyphal formation is required for virulence came from a genetic tour de force that involved creation of a defined hypha-defective mutant strain and the demonstration that the strain was attenuated in a disseminated infection model (Lo et al., 1997). The story is particularly relevant in the context of this book because the work relied heavily on high-powered genetics of a model fungus, in that case the yeast *S. cerevisiae*. Starvation induces filamentous pseudohyphal growth in *S. cerevisiae*, and the Fink lab had found that mutations in the mating-response mitogen-activated protein kinase (MAPK) pathway reduced but did not abolish the capacity for filamentation (Lo et al., 1997). This MAPK pathway ultimately activates the transcription factor Ste12. A second transcription factor, Phd1, had been identified through a high-copy-number screen for enhancers of filamentous growth (Gimeno and Fink, 1994). It seemed likely that Phd1 might represent a broadly conserved family of morphogenesis regulators, because it had substantial homology to *A. nidulans* StuA, a regulator of two polar cell divisions during conidiophore development. However, a *phd1* mutation caused no apparent defect in *S. cerevisiae* filamentation (Gimeno and Fink, 1994). Fink and colleagues considered the possibility that Phd1 and Ste12 might act in independent pathways to promote filamentous growth, so that defects in one regulator could be partially or wholly compensated for through the activity of the other. This model predicted that a *phd1 ste12* double mutant would be completely defective in filamentous growth, as was indeed found to be the case (Lo et al., 1997). This finding immediately shed light on the situation in *C. albicans*. Its Ste12 ortholog Cph1 had been studied for some time. The *C. albicans cph1/cph1* mutant had a hyphal developmental defect, but it was manifested only under some growth conditions (Liu et al., 1994). Notably, hyphal formation in serum was essentially intact in the mutant. *C. albicans* was found to have a Phd1 ortholog called Efg1, and an *efg1/efg1* mutant had a severe but not absolute defect in hyphal formation. As predicted from the findings obtained for *S. cerevisiae*, the *C. albicans efg1/efg1 cph1/cph1* double mutant had a severe hyphal formation defect, particularly in serum at 37°C (Lo et al., 1997). As predicted from the hypothesis that hyphal formation is required for virulence, the *C. albicans efg1/efg1 cph1/cph1* double mutant was very severely attenuated in a mouse disseminated infection model (Lo et al., 1997).

This work has served as a cornerstone for the analysis of *C. albicans* virulence. For example, almost every infection

model has been tested with the *efg1/efg1 cph1/cph1* double mutant; attenuation of the mutant argues for validation of the infection model. But more importantly, the strain has also opened the door for dissection of the mechanistic basis of virulence. Here, the fact that Efg1 and Cph1 are transcription factors might be viewed as a weakness, because they act indirectly to promote virulence (Kobayashi and Cutler, 1998). They are not true virulence factors. On the other hand, the inference that their cellular activity is almost certainly to alter gene expression points the way toward virulence factor identification through functional analysis of Efg1- and Cph1-regulated genes.

Let us return to the question of whether hyphal formation is required for virulence. The argument has been made that because Efg1 and Cph1 are transcription factors, they may govern all sorts of functions related to growth in vivo that have only a trivial connection to virulence (Kobayashi and Cutler, 1998). This argument is both general and valid—until one arrives at a mechanistic explanation, one must entertain all kinds of ideas about how genotype is connected to phenotype. There was one observation that seemed to rule out a trivial growth defect as the basis for the double mutant's virulence defect: Lo et al. were able to culture *C. albicans* from tissues long after infection with the double mutant, so it seemed to be capable of survival (Lo et al., 1997). This point has been made more elegantly through the use of a strain with doxycycline-regulated hyphal formation (Saville et al., 2003). The strain, referred to as "*tet-NRG1*," has a transcriptional repressor that blocks hyphal formation fused to a modified *tet* promoter that is repressed by presence of doxycycline. Hence, the natural induction of hyphal formation during growth in vivo is prevented unless doxycyline is administered to infected animals. Notably, cell density of the *tet-NRG1* strain in infected tissues was similar whether the animals received doxycycline (and hence were fated to lethal infection) or not (Saville et al., 2003). This superb study reinforces the conclusion that hyphal formation is not required for *C. albicans* to grow and survive in infected animals. Thus, it is clear that the hyphal development program is required for the organism's pathogenic potential to become manifested during disseminated infection. Admittedly, though, the contribution of cell shape in particular remains unresolved.

The study of virulence regulators may seem like a roundabout way to learn about virulence, because one is of necessity at least one step removed from the relevant mechanism. However, when one considers that virulence is multifactorial at several levels—there are diverse functions that contribute to virulence, along with numerous gene families that specify virulence factors—then a regulatory mutant may be more likely to have a pronounced virulence defect than a single-gene virulence factor mutant. One can then use the virulence regulator as a starting point to identify target genes that specify virulence factors.

Morphogenesis Triggers

Signals can function to trigger responses through multifaceted cascades in the cell. Triggers that activate different forms of *C. albicans* vary greatly within the human host. As well as signals such as amino acid availability and ammonium, nitrogen, and CO_2 concentrations, discussed below, factors such as pH and temperature are important triggers. In the laboratory we can select conditions whereby cells grow mainly as yeast form cells or primarily as hyphal cells. One must keep in mind that these signals are in an in vitro laboratory situation and therefore do not always represent the complexities of the interwoven and interconnected signaling networks present in an in vivo situation.

It is very much accepted at this point that signaling pathways are complex and environmental signals or activators that elicit changes in form may be intricate. Berman and Sudbery outlined the triggers that generate different morphologies in *C. albicans* in vitro (Berman and Sudbery, 2002). Yeast cells grow below 30°C and at pH of 4.0. A temperature of 35°C and a pH of 6.0 or a nitrogen-limited growth solid medium triggers pseudohyphal growth. Hyphal growth can also be activated in serum at temperatures of >34°C, in Lees medium at 37°C, and at a pH of 7 at 37°C. Filamentation may also be induced in spider media (commonly used for in vitro biofilm assays). Engulfment by macrophages, mouse kidneys, growth in agar matrix, iron deprivation, anoxia, and *N*-acetylglucosamine are additional inducers of filamentation (Berman and Sudbery, 2002).

Sensing and Signaling Pathways

In order to respond and adapt to triggers, cells must *sense* their environment and react accordingly. Sensors and receptors are essential for recognizing environmental changes. The adaptive response to specific stimuli (e.g., morphogenesis) is imperative for the existence of the pathogen. Each niche or microenvironment that the pathogen encounters must be sensed and adapted to.

C. albicans senses amino acid availability, and this regulates its morphogenesis. Cells can be induced to filament by amino acids. Gap1, an amino acid sensor and transporter that controls protein kinase A (PKA) targets, may be important in instigating morphogenesis signal transduction pathways. A *GAP1* mutant has defects in filamentation in GlcNac medium but not in the presence of serum (Biswas et al., 2007). This group also highlights that GlcNac stimulates expression of the *GAP1* gene and induction is reliant on *CPH1*, which they advise could place Gap1 as part of the MAPK pathway (Biswas et al., 2007). Similarly, Csy1, which stimulates cleavage of the transcription factors Stp1 and Stp2, has been implicated in amino acid sensing. Loss of Csy1 has been correlated with a change in colony morphology and hyphal formation under certain conditions (Biswas et al., 2007). Gpr1 activates the PKA pathway and senses the amino acid methionine, mediating a methionine-induced switch between yeast and hyphal forms (Maidan et al., 2005).

Morphogenesis may also be regulated by sensing of nitrogen, in which Gcn4 plays a role. Gcn4, a transcription factor, has been shown to be pivotal in response to amino acid starvation both by inducing morphogenesis and by metabolic responses (Tripathi et al., 2002).

Sensing of ammonium by Mep2, which functions as a receptor for filamentation induction in nitrogen starvation circumstances, is believed to take place through both the MAPK and the cyclic AMP (cAMP)-PKA pathways. Phosphorylation by PKA is required for Mep2 to induce pseudohyphae (Biswas et al., 2007).

C. albicans can sense and respond to carbon dioxide. The ability to sense changes in carbon dioxide concentration is vital for survival in different areas of the human host with differing CO_2 levels. Elevated levels of CO_2 of 5%—similar to those found in blood—induce filamentation and invasion in vitro through effects on adenyl cyclase (Klengel et al., 2005; Biswas et al., 2007).

The pathogen has evolved to have a sophisticated advanced response network of signaling pathways that promotes coordinated prompt morphology changes. Signaling pathways are somewhat specialized in terms of the varied

environmental signals that support morphogenesis. There are many signaling pathways that act as both positive and negative regulators of morphogenesis.

Regulators of Morphogenesis

EFG1, *CPH1*, and *CZF1* are positive regulators of the yeast-to-hypha switch. Mutations in the cAMP element *EFG1* abolish filamentation under many inducing conditions; reduced filamentation is observed in MAPK *CPH1* transcription factor deletions. As discussed above, the double deletion of *EFG1* and *CPH1* yields an avirulent strain that is nonfilamentous under most laboratory conditions. These transcription factors have additional biological functions: *EFG1* plays a role in the opaque, hyphal, and chlamydospore forms (Whiteway and Bachewich, 2007). *CPH1* functions in mating-projection formation (Chen et al., 2002; Magee et al., 2002).

CZF1 is also a positive regulator of the switch to the hyphal state (Brown et al., 1999). It is particularly noteworthy in having been discovered through a gene overexpression screen in *C. albicans*, the first such screen of which we are aware (Brown et al., 1999). Although mutations in the *CZF1* gene yield cells with reduced or slightly defective filamentation capability, a double elimination of *CZF1* and *CPH1* generates a marked filament-defective strain under agar-embedded growth conditions (Brown et al., 1999).

TUP1, *NRG1*, and *RFG1* are considered negative regulators of the yeast-to-hypha switch (Braun and Johnson, 1997; Kadosh and Johnson, 2005). The transcriptional repressor *TUP1* in both *S. cerevisiae* and *C. albicans* in conjunction with DNA binding associates *NRG1* and *RFG1* (or *ROX1* in *S. cerevisiae*) inhibits the morphology switch. These observations imply hyphal cells to be less virulent than yeast cells. While this may be accurate, one must consider other possible explanations including whether hyperfilamenting cultures disseminate well when inoculated into the bloodstream (Gow et al., 2002). Among additional functions, *TUP1* is associated with the opaque state, *NRG1* is correlated with chlamydospore formation, and *RFG1* is correlated with the pseudohyphal phase.

Other regulators of the hyphal state include *UME6*, *MCM1*, *FLO8*, *HAP5*, *EFH1*, *CPH2*, *ASH1*, *TEC1*, and *ACE2* (Whiteway and Bachewich, 2007). Other regulators of the pseudohyphal state include *FKH2*, *TCC1*, *SSN6*, *ACE2*, and *RAP1* (Whiteway and Bachewich, 2007).

pH-Mediated Regulation

RIM101 specifies a conserved transcription factor that mediates pH responses in diverse fungi (Penalva and Arst, 2004). *C. albicans RIM101* is required for neutral/alkaline pH to induce hyphae (Davis, 2003). It is correlated with the hyphal and pseudohyphal states. Under alkaline conditions, Rim101 is activated by carboxyl-terminal cleavage (Baek et al., 2006). *RIM101* activity is stimulated, alkaline pH-induced genes are activated, alkaline pH-repressed genes are suppressed, and the result is filamentation.

Cyclins and Morphology Changes

Cyclins play a major role in *C. albicans* morphogenesis (Berman and Sudbery, 2002; Sinha et al., 2007; Whiteway and Bachewich, 2007). Succinctly, Ccn1 is required for maintenance of hyphal growth. Cln3 is needed for budding. But neither of these is required for initiation of the hyphal form. Rather, Hgc1 is necessary for hyphal development. Whiteway and Bachewich conclude that these cyclins, all G_1-type cyclins, evoke a relationship between G_1 cyclins and regulation of morphogenesis (Whiteway and Bachewich, 2007). The B-type cyclins Clb4 and Clb2 as well as the protein kinase Cdc28 also control morphogenesis but not the yeast-to-hyphal-form switch (Whiteway and Bachewich, 2007).

Phenotypic Switching and Morphology Changes

Many *C. albicans* strains undergo epigenetic morphological switching, typically manifested as distinct colony morphologies (Soll, 1992). Among these, the best known is the white-opaque switch (Slutsky et al., 1987), which involves white oval cell colonies switching to opaque oblong cell colonies with raised dots on the surface, where cells are twice the size of the white cells (Sudbery et al., 2004). The white-opaque switch had long fascinated the *C. albicans* community, but the landmark discovery that opaque cells are the mating-competent form of *C. albicans* (Miller and Johnson, 2002) placed this bizarre epigenetic switch firmly in the foreground of basic *C. albicans* research questions. The opaque state is controlled by the Wor1 transcription factor, which dictates cell type through an autoregulatory circuit (Zordan et al., 2007). White-opaque switching, as well as the other less well understood switching systems, are further instances where the pathogen morphs from one state to another for the potential purpose of giving itself an adaptive advantage. In the case of white-opaque switching, it is not aiming to express a virulence factor for invasion of the host but employing a switch for the purpose of mating. Morphogenesis to the opaque form is imperative for mating (Whiteway and Bachewich, 2007).

Further Investigation of the Morphogenesis-Pathogenesis Connection

Many, including Biswas et al. (Biswas et al., 2007), suggest that studies of morphogenesis have traditionally been hindered by a lack of genetic tools to investigate large-scale genetic screens. Such tools and mutant libraries are now available, though, so this limitation can be readily addressed (Noble and Johnson, 2007).

To assess the role of morphogenesis and signaling and its contribution to virulence, one must assess gene expression. The timing of morphogenesis at different stages of infection will be important. According to Whiteway and Bachewich, direct relationships between altered gene expression and concurrent cell shape changes have not been established (Whiteway and Bachewich, 2007). This is clearly an important area of ongoing investigation. It appears, however, as argued by Gow and colleagues (Gow et al., 2002), that no straightforward, broad-ranging, or universal relationship can be established between morphology and invasive capability.

In terms of virulence, we will have to distinguish between contributions from cell morphology and gene expression and ask whether the two are always linked or complementary or whether there is a cause-and-effect situation in operation. Undoubtedly both, either together or individually, bestow on the organism the ability to survive in a variety of niches. Hypha-specific genes clearly are the target for such research.

Many of the morphogenesis signals mentioned earlier govern cell wall structure, and this is an area for further investigation. The cell wall is mainly comprised of β-1-3 glucan and is structurally and functionally very important to the cell, not only for adherence, integrity, sensing, and interaction but also for morphogenesis. Its plasticity is key to morphogenesis. Growth conditions, signaling, and morphogenesis affect the cell wall, and links between cell wall and morphogenesis are open to development.

Whiteway and Bachewich point out that, on a transcriptional level, signals can be responded to by expression regulation (Whiteway and Bachewich, 2007). Key parts of signaling pathways are the kinases, and these regulate transcription factors and other cell types.

In the future, interesting approaches such as that of Kaufmann and colleagues (Kaufmann et al., 2007), who found an epigenetic phenotype to be heritable and the stability of the phenotype to also be heritable, may provide novel insights into morphological changes that facilitate the C. *albicans* pathogenic lifestyle.

ADHESION

The outer surface, which has contact with the environment and host, is the crucial sensor, protector, mediator, and barrier point for the pathogen. Surface interactions between C. *albicans* and its environment, and often specifically with a medical device, are of great consequence. From a medical perspective, targeting the cell wall for drug design and development is promising. Indeed, a major group of antifungals, the echinocandins, inhibit β-1,3-glucan synthesis and thus target the interaction interface.

A large proportion of the cell wall is comprised of carbohydrates. The interplay between constituents that generate the cell wall structure and the architecture is cemented by hydrogen bonds, hydrophobic interactions, and covalent bonds (Chaffin et al., 1998). Surait et al. found glycosidic linkages between glucan and chitin in the nascent cell wall (Chaffin et al., 1998). Mannoproteins may covalently link to β-glucans. The β-1,3- and β-1,6-glucans are linked to proteins through phosphodiester linkages (Chaffin et al., 1998). Proteins can be N or O linked to polysaccharides via asparagines or serine/threonine, respectively (Sohn et al., 2006).

From 6 to 25% of the cell wall is composed of protein. Sohn et al. argue that this component is a primary distinguishing factor between the cell walls of pathogenic and nonpathogenic fungi (Sohn et al., 2006). In addition, the cell wall protein constitution differs in different cell morphologies (Sohn et al., 2006). These facts lead us to our focal point, cell wall proteins as pathogenic determinants, for this section of the chapter. The roles of cell wall proteins in adherence are most closely tied to virulence at this time; thus, adherence is our main theme.

There are three main classes of cell wall proteins: proteins with internal repeats (Pir), noncovalent or secreted proteins, and glycophosphatidylinositol (GPI) proteins. Thus far, the GPI proteins are most clearly implicated in having direct roles in adherence, though there remains much to learn about cell wall protein function.

The Pir proteins are highly *O*-glycosylated proteins with internal repeats that are linked to the cell wall by β-1,3 glucans and lack a GPI anchor (Kandasamy et al., 2000). Kandasamy et al. found the Pir proteins of C. *albicans* to be related to those of *S. cerevisiae*, specifically Hsp150 of *S. cerevisiae* (Kandasamy et al., 2000). Ruiz-Herrera and colleagues noted that C. *albicans* has a single Pir-encoding gene and that two Pir proteins are produced by dissimilar alleles (Ruiz-Herrera et al., 2006). Chaffin (Chaffin, 2008) and Ruiz-Herrera et al. (Ruiz-Herrera et al., 2006) suggested that failure to obtain a homozygous *PIR1* mutant suggests it is essential. Chaffin (Chaffin, 2008) suggested that Pir proteins contribute to the overall architecture of the cell, based upon studies indicating that expression of *PIR1* is repressed by Rim101 (hence a connection to pH) and can be regulated by Efg1 and have reduced expression in hyphae.

Noncovalent and secreted proteins are extracellular and either are loosely associated with the cell wall or simply travel through it. There are numerous cell wall-localized proteins of this class including Sun41, Eng1, and Bgl2 (all comprehensively reviewed by Chaffin [2008]). These proteins may contribute to cell wall structure directly, as building blocks, or more indirectly, through enzymatic remodeling activities. Among secreted proteins, the secreted aspartyl proteases have received substantial attention because they are expressed during infection and can modify cell surfaces, thus contributing to diverse aspects of infection (Naglik et al., 2003, 2004). In addition, several secreted aspartyl proteases are expressed preferentially during hyphal growth and may thus be components that connect morphogenesis to pathogenicity (Naglik et al., 2003, 2004).

GPI Proteins

GPI-anchored proteins are found in all eukaryotic cells (Richard and Plaine, 2007). They are thought to connect with lipid rafts found in human cells. The GPI proteins of C. *albicans* are linked to the β-1,6 glucans, and for many, their expression can vary depending on morphology. As in previous sections of this chapter, we frequently look to *S. cerevisiae* to steer our thinking. The characterized GPI proteins of the nonpathogen appear mostly involved in cell wall biosynthesis, flocculation, protease activity, sporulation, and mating (Richard and Plaine, 2007). Interestingly, *S. cerevisiae* and *Schizosaccharomyces pombe* have fewer GPI protein genes than C. *albicans* (Richard and Plaine, 2007). Of particular interest to us is the finding that 11% of the GPI proteins in C. *albicans* are thought to have a function in cell-to-cell adhesion (Richard and Plaine, 2007). Deletion of proteins key to GPI biosynthesis is lethal, and GPI anchors are essential for viability in yeast (Richard and Plaine, 2007).

GPI-anchored proteins possess conserved features that facilitate gene identification. They have an N-terminal signal sequence that directs cotranslational secretion into the lumen of the endoplasmic reticulum, a C-terminal hydrophobic domain for transient attachment to the endoplasmic reticulum membrane, and an ω-site, where the protein is cleaved for ligation to the GPI anchor (Richard and Plaine, 2007). The GPI anchor has a lipid group, an *N*-acetylglucosamine group, three mannose groups, and a phosphoethanol amine group. These connect the anchor and the protein by amid linkage (Richard and Plaine, 2007). The anchor may target the protein to the cell wall or membrane (Chaffin, 2008) and can be removed by specific phospholipase action (making the protein water soluble). However, the signature feature of GPI anchors is that they enable transfer of the protein, along with a GPI anchor remnant, to a covalent linkage with cell wall β-glucan.

Richard and Plaine combined data sets from several research groups (Richard and Plaine, 2007) to come up with 115 GPI proteins in the C. *albicans* genome, representing 25 protein families. They define four functional categories: unknown function (76 genes), cell wall biogenesis or remodeling (15 genes), cell-cell adhesion and interaction (13 genes), and enzymatic functions (11 genes). Richard and Plaine (Richard and Plaine, 2007) pointed out that much of the novel discoveries for *Candida* may lie in the 76 gene products with unknown function for two reasons: (i) GPIs are at the cell surface and therefore could be important to interactions, e.g., with the host or the environment; and (ii) *Candida* is highly evolved to tolerate and adapt to its environment, and this possibly stems from the action of some of these 76 gene products.

Forty-one of the 76 genes of unknown function are found to be regulated by a number of transcription factors and conditions. Notably, Sohn et al. asserted that apposite regulation of the cell surface is required for adhesion and thereby for host colonization (Sohn et al., 2006). In this case, nine of them are regulated by Rim101 and also controlled by iron level variation, morphogenetic switching, Nrg1, and Tup1 (Richard and Plaine, 2007).

ALS Family

The *ALS* (agglutinin-like sequence) gene family specifies a family of GPI proteins. The *ALS* genes are similar to the alpha-agglutinin genes of *S. cerevisiae*, have eight members, and are found at the cell surface. *ALS1*, *ALS2*, *ALS3*, and *ALS4* are similar, while *ALS5*, *ALS6*, and *ALS7* (*ALS6* and *ALS7* are associated with low-level expression) are grouped together and *ALS9* is grouped on its own (Chaffin, 2008). The Als family is characterized by conserved internal tandem repeats (Hoyer, 2001). Many Als proteins have been found to be localized to the cell surface, and this positioning suggests they are at the interface for interaction.

Variability in size is characteristic of the ALS family (Hoyer, 2001). The root of variability is principally the tandem repeats' middle section. This yields huge variation. Zhang et al. described 60 diverse forms of *ALS7* that were found among 66 *C. albicans* strains studied (Zhang et al., 2003). It is postulated that variation in the intragenic repeats is a mechanism for the cell to escape host defenses (Richard and Plaine, 2007). This may link to ability to deceive the immune system (Verstrepen et al., 2004). The repeat region permits recombination so that gene reorganization can occur, enabling cell surface proteins with new functions.

Als3 is required for virulence-associated phenotypes in various models. Zhao et al. showed that an Als3 mutant cannot adhere to endothelial or epithelial cells (Zhao et al., 2004). In addition, *C. albicans* required either Als3 or the closely related Als1 in order to form biofilms in an in vivo catheter infection model (Nobile et al., 2008a). Interestingly, overexpression of *ALS6*, *ALS7*, or *ALS9* in an *als1*Δ/Δ *als3*Δ/Δ double mutant restored biofilm formation in vivo substantially (Nobile et al., 2008a), providing evidence for functional redundancy in this gene family. Evidence for functional redundancy also comes from Sheppard et al. (Sheppard et al., 2004), whose analysis of Als proteins expressed in *S. cerevisiae* revealed that Als1, Als3, and Als5 all confer ability to bind to gelatin, fibronectin, laminin, FaDU epithelial cells, and endothelial cells. Als6, Als7, and Als9 all had more selective or limited binding ability in these assays (Sheppard et al., 2004).

Studies by Filler and colleagues have addressed the function of Als3 in mechanistic detail. They identified mammalian cadherins as specific receptors for Als3 (Phan et al., 2007). Remarkably, Als3 does not simply bind to surface cadherin but confers uptake ability as well. In other words, Als3 functions as an invasin to permit *C. albicans* to induce its phagocytosis by host cells (Phan et al., 2007).

Hwp1

The de novo discovery of the hypha-specific cell wall protein Hwp1 by Staab et al. (Staab et al., 1999) contributed significantly to the concept of adhesion and its relationship to hyphal morphogenesis. Strains lacking Hwp1 did not attach stably to buccal epithelial cells (found to be the substrate for the protein) and had decreased virulence in the mouse candidiasis model. The Hwp1 protein is covalently attached to cell surface proteins of the host via a host transglutaminase (Sohn et al., 2006). *HWP1* is regulated by the cAMP/PKA pathway (via Efg1and Rim101) (Sohn et al., 2006). The protein has been shown to be proline rich, and Chaffin et al. (Chaffin et al., 1998) proposed that it may share similar functions with other proline-rich proteins such as maintenance of polypeptide chains in extended conformations as well as mediating noncovalent interactions between chains.

Hwp1 seems to have a distinct function in biofilm formation. It is required for biofilm formation in an in vitro model (Nobile et al., 2006b) that lacks host cells or host-derived transglutaminase activity. It is also required for biofilm formation in an in vivo catheter infection model (Nobile, 2006b); in that case, a possible role for host-derived transglutaminase has not been ruled out.

Eap1

Eap1 protein was originally identified through a heterologous expression screen, using *S. cerevisiae* as a host, in a search for *C. albicans* adhesins (Li and Palecek, 2003). (A similar strategy had been used some time before to identify Als1 [Fu et al., 1998b].) Eap1 has also been analyzed in considerable detail and was found to promote both cell-substrate and cell-cell adherence (Li and Palecek, 2008). Eap1, like Hwp1 and Als1/3, is required for biofilm formation in vivo in the catheter infection model (Li et al., 2007).

Hyr1

Hyphally regulated Hyr1 orthologs or *IFF* genes are still understudied relative to some of the other families. Ten of the 12 genes in the family have GPI anchors, while 2 genes, *IFF10* and *IFF11*, do not (Richard and Plaine, 2007). On phylogenetic analysis, these two were found to diverge from the rest of the group, and this was illustrated nicely by Richard and Plaine (2007). Chaffin et al. (Chaffin et al., 1998) suggested that the characteristic N-terminal signal sequence, the C-terminal GPI signal sequence, and the serine-threonine-rich area are similar to those of many in *S. cerevisiae*, and gave examples such as Cwp1, Tip1, and Flo1.

Bailey et al. (Bailey et al., 1996) showed that *HYR1* is expressed under hyphal growth conditions but not under yeast growth conditions. Mutations disrupting both alleles of *HYR1* displayed no obvious phenotype under conditions studied. It is postulated that this nonessential protein may play a role in structure, more so than in adhesion, but due to structure cannot be discounted from having attachment capabilities.

Other Proteins with Adherence Capability

Our survey of *C. albicans* adhesins above is by no means comprehensive. We have focused on proteins whose mechanisms of action have been addressed or, in the case of Hyr1, have perplexed the *C. albicans* community for some time. Additional proteins, e.g., Ywp1, Ecm33, Pra1, and Mp65, have been implicated in adhesion (Chaffin, 2008). In addition, Rbt5 and related CFEM (acronym for "common in several fungal extracellular membrane proteins") protein family members have been implicated in adhesion through in vitro biofilm formation assays (Perez et al., 2006) and through host cell binding assays (Nobile et al., 2008b). There clearly remains much to learn about the diverse GPI proteins and their functions in the context of adhesion.

Medical Impact of Adhesion

The ability to adhere is a pathogenic determinant. Richard and Plaine summarized their data and found that of the 15 mutants created from the 115 GPI proteins, 12 exhibited reduced virulence and 3 had wild-type virulence (Richard

and Plaine, 2007). This supports a potential correlation between virulence and GPI/cell wall proteins. However, the authors attached a caveat that the sample size was too small to be of statistical significance.

From a medical perspective, adhesive capability is a virulence determinant not only in conferring attachment to host tissue but also for its role in biofilm formation. A biofilm is a population of either single or multiple species growing on a surface and usually embedded in an extracellular matrix. The development of a biofilm requires adherence, matrix assembly, and quorum sensing. Adherence is usually to an exogenous substrate, either the human host or a medical implant (reviewed by Blankenship and Mitchell, 2006).

ACE2 of *C. albicans* has been found to be similar to *ACE2* in *S. cerevisiae*, and through mutant analysis deletion strains have been found to have diminished adherence. Additionally, they are reported to produce biofilms notably different from those of wild-type strains and are avirulent in mice (Kelly et al., 2004).

The Als proteins and Hwp1 mentioned earlier are key molecular players in biofilm formation. Following identification of the transcription factor Bcr1 as playing a role in biofilm formation and its regulation by Tec1 (Nobile and Mitchell, 2005), the Bcr1 zinc-finger transcription factor was identified as controlling Als1, Als3, and Hwp1. It is thought to contribute to hyphal development and stimulate expression of the aforementioned genes. Through employment of the rat venous catheter model, Hwp1 was established to partake in biofilm formation both in vitro and in vivo, whereas in vivo assays using the rat venous catheter model showed Als3 to be essential primarily in vitro (Nobile et al., 2006a). Blankenship and Mitchell hypothesized that these adhesins are likely to play a greater role in hypha-hypha or hypha-yeast adherence than in initial attachment to the surface (Blankenship and Mitchell, 2006). To further the understanding of the Als proteins in adherence and biofilm construction, Nobile et al. showed that overexpression of Als proteins in biofilm-defective strains (lacking Als1 or Als3) facilitated biofilm development (Nobile et al., 2008a). Most interesting was their finding that a combination of biofilm-defective strains (lacking Als1, Als3, or Hwp1) yielded a "hybrid biofilm" in vitro and in vivo (Nobile et al., 2008a).

On reflection, the targeting of the cell wall and its components for novel drug development looks auspicious. A number of studies reviewed by Chaffin (Chaffin, 2008) show that treatment with caspofungin was associated with a decrease in adherence to extracellular matrix. Also highlighted was a study showing that five of six fluconazole-sensitive strains had reduced adherence to extracellular matrix and three strains had reduced adherence to fibronectin.

The adhesins appear to be a good target also for vaccines, since disabling their function can inhibit adherence and thereby diminish invasion of the host environment, either tissue or implanted devices.

In a series of publications, groups at the University of California–Los Angeles have identified and developed vaccines using the Als1 protein. Ibrahim et al. showed that immunization with the recombinant domain of Als1p yielded increased survival of BALB/c mice when they were later challenged with lethal levels of *C. albicans* (Ibrahim et al., 2005). The group found the immunization to protect in B-cell-deficient mice but not in T-cell-deficient mice, leading them to conclude that the vaccine stimulates the cell-mediated immune system. The group furthered this work by showing subcutaneous vaccination to be more efficient than their previous intraperitoneal approach (Spellberg et al., 2005). Building on this, the researchers showed the vaccine to be effective against several *Candida* strains including *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* (Ibrahim et al., 2006). Vaccination with a recombinant Al3p elicited a broader antibody response than the Als1 vaccination. The Als3p-based immunization proved to be as effective as its Als1 predecessor and more effective in disseminated candidiasis (Spellberg et al., 2005).

Targeting of the adherence virulence factor in the hope of developing vaccines and innovative therapies to prevent invasive candidiasis looks promising.

AZOLE RESISTANCE

The most common antifungal therapies for candidiasis include caspofungin, fluconazole, and amphotericin B. Currently antifungal treatments are limited, and emerging drug resistance, particularly to the azoles, is a cause for concern. Azole resistance is increasingly seen even in non-AIDS patients. New novel antifungal treatments are being developed, but worry about resistance stems from inherent knowledge of antibiotic resistance in bacteria. Hence, it is essential that we begin to understand the mechanisms underlying resistance and develop new preventative and treatment options.

White et al. suggested the azoles, which are fungistatic, to be advantageous because of their "relative safety and ease of delivery" (White et al., 1998). The azole voriconazole is currently active against all species of *Candida* (Kauffman, 2006). Therapies such as fluconazole are not active against *C. krusei* (Kauffman, 2006). *C. guilliermondii* and *C. rugosa* invasive candidiasis infections have emerged as problematic due to decreased susceptibility to fluconazole (Pfaller and Diekema, 2007). Of concern is the increase in *C. glabrata* infections, possibly linked to the resistance of the organism to fluconazole (Kauffman, 2006). *C. inconspicua* and *C. norvegensis* demonstrate inherent fluconazole resistance (Pfaller and Diekema, 2007).

Resistance can be characterized by therapy failure or requirement for increased antifungal MIC. White et al. advised that clinical resistance has been defined by persistence or progression of infection in spite of the administration of correct antimicrobial therapy (White et al., 1998). The ability to overcome antifungal treatment contributes to the ability to be virulent. Emerging azole resistance may stem from inappropriate therapy choice, incomplete treatment, transmission of resistant strains, or unforeseen stimulation of resistance. Severe immunosuppression is a risk factor for resistance development, as is prior exposure to the azole. Success of antimicrobial therapy is dependent on the pathogens' vulnerability and also on host immune response, drug diffusion and distribution, patient conformity to therapy, and removal of infecting elements, e.g., a catheter (White et al., 1998). The stability of the resistant strain and ability to transmit also contribute to resistance perseverance.

From a clinical perspective, emerging fluconazole and amphotericin B resistance is of concern. Nolte et al. discussed the emerging problem as disconcerting in their patient population (Nolte et al., 1997). They reported on two leukemia patients who acquired infection by resistant strains after fluconazole prophylaxis treatment. The strains were also resistant to other azoles, and membrane sterol changes were observed. The deficiency in ergosterol in the cytoplasmic membrane was hypothesized to contribute to the resistance to amphotericin B, which ultimately had to be administered in high doses in combination with flucytosine for successful treatment. White et al. reported on three

studies which estimated azole resistance at a level of 21 to 32% in symptomatic patients and up to 14% in asymptomatic patients (White et al., 1998).

Factors That Contribute to Resistance

The clinical, cellular, and molecular factors contributing to resistance have been effectively reviewed in detail by White et al. (White et al., 1998), so here we summarize some of the important contributing factors highlighted by the reviewers. White et al. list the factors contributing to resistance (as conferred by the pathogen) to include initial MIC, cell type, genomic stability of the strain, the size of the population, and biofilms (White et al., 1998). In the same table, they list the factors contributed by the drug, including the fungistatic nature of the drug (unlike the fungicidal nature of amphotericin B), dosing, drug-drug interactions, and components such as metabolism, grouped under the term pharmacokinetics. The host also contributes to resistance, in the forms of immune system, mounted immune response, infection location, infection acuteness, presence of exogenous components (e.g., catheter), and abscess formation (White et al., 1998).

It is suggested that *azole treatment* may suppress the growth of susceptible strains but allow resistant strains to thrive. Strains of *C. albicans* that are intrinsically more resistant may have acquired resistance through genetic mutations (discussed below) and prosper. Mixed infections (susceptible and resistant cultures) and strain replacement are two possibilities considered by White et al. as contributing to drug resistance. These authors reference studies that analyze (by karyotyping and restriction fragment length polymorphism) a mixture of susceptible and resistant strains in patients over time. It is proposed that changes in the balance of each may stem from azole treatment or through strain replacement. Transfer or *transmission of resistant strains* between patients also plays a role in drug resistance (White et al., 1998).

The possibility of *prolonged drug treatment* affording the opportunity for resistant cells to outgrow by selection is considered by White et al. (White et al., 1998). *Genetic mutations*, specifically when strains are under drug selection, may permit resistant strains to outcompete, and the authors say the strain will persist so long as the mutations have not negatively affected cell fitness. *Transient gene expression*, whereby a temporary change in phenotype causes cells to be spontaneously resistant (only when the drug is present), can also be a contributory factor (White et al., 1998). Finally, consideration is given by White et al. to *modifications in cell type* and in the *population as a whole*, which may have an impact on drug resistance capacity. They advocate that a strain that could switch from yeast cells to hyphae in the presence of azoles is likely more pathogenic (and resistant) than a sensitive yeast form culture, as azoles interrupt hyphal production at 1/10 of the concentration required to treat yeast form cells. In terms of whole-population contribution to resistance the reviewers envisage (i) population "bottlenecks" as an area for possible selection of resistant strains and (ii) genomic instability and variation possibly yielding drug-resistant varieties (White et al., 1998).

The Molecular Perspective

Key focal points for examining resistance have been the ATP-binding cassette transporters *CDR1* and *CDR2*. Δ*CDR1* mutants have been shown to be hypersusceptible to a number of azoles and also a number of other antifungals (Sanglard et al., 1996). Manoharlal et al. (2008) studied *CDR1* and *CDR2* to establish the potential molecular mechanisms for azole resistance. Using two resistant and two susceptible strains, the researchers used reporter fusions to show increased transcription of *CDR1* and greater stability of the *CDR1* mRNA in the resistant isolates but found protein stability to be unchanged between the isolates. Comparing the fusions to the *CDR1* promoter versus the *CDR1* open reading frame, the group reported that important sequences within the *CDR1* coding sequence contribute to greater expression in resistant strains (Manoharlal et al., 2008).

MDR1, an efflux transporter, was found to be constitutively highly expressed in many fluconazole-resistant strains (Riggle and Kumamoto, 2006). Riggle and Kumamoto identified a promoter fragment (MDRE) that is involved in *MDR1* transcription, although the group found that other upstream components were also involved in transcription of overexpressing *MDR1* strains. Additionally, the researchers found the transcription factor Mcm1 to be involved in *MDR1* expression regulation (Riggle and Kumamoto, 2006). Morschhauser et al. (2007) employed genome-wide gene expression analysis to show *MRR1*, a zinc cluster transcription factor, to be upregulated coordinately with *MDR1* in drug-resistant isolates. They found the gene targets of Mrr1 encoded oxidoreductases and hypothesized that upregulation of such components in fluconazole-resistant isolates could aid in cell damage prevention in the presence of fluconazole. Their establishing of mutations in fluconazole-resistant strains that constituted single-nucleotide substitutions in *MRR1* allowed them to establish that such mutations yielded constitutive overexpression of *MDR1*, altering the strain from susceptible to resistant to the drug treatment (Morschhauser et al., 2007)

TAC1, a zinc-finger transcription factor, was shown to regulate *CDR1* and *CDR2* (Coste et al., 2004). Their genome screen involved analysis of Zn(2)-Cys(6) finger genes, stemming from their hypothesis that such a gene, encoding a transcription factor, was a likely regulator on the basis of their observation that a *cis*-acting drug-responsive element (DRE) was contained in the promoters of both *CDR1* and *CDR2*. DRE is the site to which Tac1 binds and is discussed later in this section. The group showed that introduction of a *TAC1* allele from a resistant strain to a susceptible strain resulted in upregulation of both *CDR* genes to the point of constitutive upregulation.

TAC1 is located close to the mating-type locus (MTL). The literature suggests an association between homozygosity at the MTL locus and azole resistance (Coste et al., 2006). *TAC1* is situated on chromosome 5 and is required for the upregulation of *CDR1* and *CDR2* (Coste et al., 2006). The researchers distinguish between hyperactive *TAC1* alleles and wild-type alleles and report that wild-type alleles yield upregulation of the *CDR* genes on induction. Hyperactive alleles yield constitutive high expression of the genes. Interestingly, comparative genome hybridization and single nucleotide polymorphism arrays showed that loss of *TAC1* heterozygosity can arise by recombination of portions of chromosome 5 or by duplication of chromosome 5. Homozygosity at MTL is associated with *TAC1* homozygosity by at least two mechanisms, but MTL homozygosity does not play a role in azole resistance (Coste et al., 2006).

cis- and *trans*-acting effectors are important in analysis of transcriptional control. *trans*-acting factors regulating *CDR1* and *CDR2* were reported by Chen et al. (Chen et al., 2004). They explained a possible *CDR1* activator during a screening of a *C. albicans* genomic library expressed in an *S. cerevisiae* strain. This factor is *NDT80*, and its deletion yields hypersensitivity to the azoles and reduced inducible expression of *CDR1* (Chen et al., 2004).

As well as the DRE present in the promoters of both *CDR1* and *2,* there is a basal response element in the *CDR1* promoter, which regulates *CDR1* expression (Coste et al., 2006). Additionally, a second basal response element and one negative regulatory element have been found in the *CDR1* promoter. Coste et al. (Coste et al., 2006) say that Karnani et al. (Karnani et al., 2004) identified SRE1 and SRE2 (steroid response elements) for the *CDR1* gene.

Selmecki et al. (2006), using a modified comparative genomic hybridization method, found aneuploidy to be seven times more common in fluconazole-resistant strains. Such aneuploidy, advocated by the researchers to be mainly trisomy, was predominantly associated with chromosome 5 (Selmecki et al., 2006). The group found the isochrome formed around a single centromere flanked by inverted repeats. Acquiring of the isochrome correlated with increased fluconazole resistance, and loss of the isochrome was linked to decrease in resistance. The isochrome in question was found to contain many genes associated with azole drug targets including efflux pumps and a transcription factor, e.g., *ERG11* and *TAC1*. It is reported that mutations in the coding sequence of *ERG11* that modify the affinity of the azoles for their target or upregulate *ERG11* increase azole resistance (Coste et al., 2007). Selmecki et al. furthered this observation in a 2008 study in which comparative genome hybridization was employed, to find that two additional copies of Chr5L (chromosome 5) were present on the isochrome, and this bestowed greater fluconazole resistance (Selmecki et al., 2008). Similarly, strains with four copies of Chr5L had a higher MIC requirement of fluconazole than strains with only two copies (although the authors acknowledge that other genetic differences between the strains do not allow for direct comparative analysis). They successfully partially truncated Chr5L, which lessened fluconazole resistance. It was found that the primary route by which the isochrome contributes to enhanced azole resistance is via the genes *TACI* and *ERG11*. These two genes were found to contribute independently but had an additive effect on resistance capacity (Selmecki et al., 2008). Showing that azole resistance stems from mutations in certain genes as well as from more copies of each being present, Coste et al. (2007) employed comparative genome hybridization and karyotyping to demonstrate the role of "gain of function" and "loss of heterozygosity" in resistance. Gain of function was found only in hyperactive *TAC1* alleles where one of either three amino acid substitutions or one of two amino acid deletions had taken place.

As well as *ERG11,* other *ERG* genes are likely to play a role in drug resistance by modification of target enzymes or the ergosterol biosynthesis pathway. White et al. highlighted *ERG3* in this regard, referencing work showing mRNA levels of *ERG3* to be increased in *ERG11* deletions and vice versa. They concluded that there is transcriptional control of these genes in the pathway and speculate that it is by negative feedback from ergosterol, as a deficiency in ergosterol due to deletions in either *ERG3* or *ERG11* induces transcription of the other ERG gene (White et al., 1998).

Considerations for Future Therapeutics

The increasing prevalence of resistant isolates to the newer azole class of antifungals suggests that future antifungal treatment and prophylaxis may be challenging. Currently, the echinocandins such as caspofungin are as successful in treating candidiasis as amphotericin B (Kauffman, 2006), but preparations must be made for the possibility that resistance to this treatment may develop.

Berman and Sudbery articulate that *C. albicans* has many biological processes similar to those of humans and antifungal treatments can therefore have harmful side effects; hence, drugs are used at fungistatic rather than fungicidal concentrations. They advocate an imperative aim of research in this area to be the identification of apposite targets for antifungal tools (Berman and Sudbery, 2002).

A promising tactic is to pursue *C. albicans* virulence factors. Discovery of genes that govern virulence factors allow for identification of gene products that could be targets for therapy. An illustration comes from the development of adhesin Als3 as a therapeutic target (Ibrahim et al., 2005, 2006; Spellberg et al., 2005). These may also provide an approach for diagnostic targets and tools. Responses to host factors also provide a wealth of knowledge on the in vivo situation and generate new pathway targets not only of the pathogen but also of pathways of interaction between host and pathogen that could be exploited for pharmaceutical objectives. Until new targets are identified for pharmacological utilization, consideration may be given to combinatorial drug administration.

PERSPECTIVE

Much of what we understand mechanistically about *C. albicans* virulence comes from studying proteins like Hwp1 and Als3 in isolation. There is a wealth of information now that connects *C. albicans* genes to virulence in one or several infection models. One challenge that lies ahead is to distinguish among metabolic functions, virulence regulators, and true virulence factors. All are interesting and have enormous therapeutic potential; mechanistic insight will allow this potential to be developed. A second challenge is to understand how virulence factors act together during the course of infection, how virulence regulators coordinate those functions, and how metabolism fuels these processes. From the therapeutic perspective, we need to understand how prospective antifungal strategies impact fungal metabolism, virulence, and its regulation. A final thought is that natural selection has probably acted on *C. albicans* most strongly as a commensal. Its functional repertoire has likely evolved to avoid inflammation of host tissues and to support effective competition with its bacterial cohabitants. The true logic behind deployment of *C. albicans* virulence factors may be most apparent when they are viewed as commensalism factors.

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