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REVIEW

Genetic Networks That Govern Sexual Reproduction in the Pezizomycotina

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SUMMARY Sexual development in filamentous fungi is a complex process that relies on the precise control of and interaction between a variety of genetic networks and pathways. The mating-type (*MAT*) genes are the master regulators of this process and typically act as transcription factors, which control the expression of genes involved at all stages of the sexual cycle. In many fungi, the sexual cycle typically begins when the mating pheromones of one mating type are recognized by a compatible partner, followed by physical interaction and fertilization. Subsequently, highly specialized sexual structures are formed, within which the sexual spores develop after rounds of meiosis and mitosis. These spores are then released and germinate, forming new individuals that initiate new cycles of growth. This review provides an overview of the known genetic networks and pathways that are involved in each major stage of the sexual cycle in filamentous ascomycete fungi.

KEYWORDS sexual reproduction, Pezizomycotina, *MAT* genes, pheromones, fertility, pheromones

INTRODUCTION

Sexual reproduction is an essential component of the life cycle in most eukaryotes. This is particularly true for metazoans, where sexual reproduction is usually the

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only process that allows for the production of offspring (1). Fungi are no exception and have maintained complex and active sexual cycles, in addition to being capable of asexual reproduction (2). Combined with the energy intensive nature of sexual development, this suggests that regular cycles of sexual development are important to the long-term survival of fungal species, resulting in benefits beyond simply the production of genetically unique offspring (3).

Sexual reproduction has been described in many species across the phylum Ascomycota (4, 5). Although the members of this group show immense diversity in morphology and ecology, their sexual cycles remain highly conserved. In most species, sexual reproduction is centrally controlled by two sets of major regulators (6): the mating-type (*MAT*) genes and the pheromone response pathway (7). While the sexual cycles of only a subset of species have been thoroughly investigated, their conserved nature, even between distantly related taxa, suggests that these processes are likely to be similar in most fungal lineages. Despite these similarities, there are some differences with regard to the functions of the genes involved and the role of external processes in the sexual cycles of fungi.

Ascomycete fungi reside in three subphyla: Saccharomycotina, Taphrinomycotina, and Pezizomycotina (8). The first two groups accommodate yeasts and dimorphic species, which have been extensively studied, particularly with regard to their sexual cycles (9, 10). In contrast, the Pezizomycotina is comprised entirely of filamentous fungi. Representing the largest subphylum and encompassing a wide diversity of important fungi, the Pezizomycotina accommodates both detrimental and beneficial fungi. Examples include the human-pathogenic opportunist *Aspergillus fumigatus* (11) and the dermatophyte *Trichophyton rubrum* (12), as well as plant pathogens such as the notorious chestnut blight fungus, *Cryphonectria parasitica* (13), and the causal agent of pine pitch canker, *Fusarium circinatum* (14). A number of ecologically important species have also been described, including those that are important for wood and litter decay, as well as those that form associations with other organisms, including lichens (15, 16).

Importantly, the subphylum Pezizomycotina also accommodates a number of important model species, including *Neurospora crassa*. Research on this species has significantly contributed to our understanding of many fundamental genetic processes and molecular mechanisms (as reviewed in reference 17). The "one gene, one enzyme" hypothesis that gave birth to molecular biology was developed using nutritional mutants of *N. crassa* in 1941. Since then, this species been used as a model to study the molecular components of circadian rhythms, the Repeat-Induced Point (RIP) mutation genome defense mechanism, RNA interference (RNAi) and epigenetics. Together with other model Pezizomycotina organisms, such as *Aspergillus nidulans*, fungi have provided insights into a variety of important eukaryotic processes, including photosensing (18), secondary metabolism (19, 20), cellular synthesis pathways (21), asexual reproduction (22, 23), and sexual development (24–27). In this review, we consider the genes and genetic networks that govern sexual reproduction in a wide variety of species from the Pezizomycotina, considering research done in both model and non-model fungi.

MATING IN FILAMENTOUS FUNGI

Mating in ascomycete fungi is relatively well conserved, particularly with regard to the genetic mechanisms that govern sexual development (28). In both filamentous fungi and the yeasts, sexual reproduction is regulated by the mating-type (*MAT*) genes, mating pheromones, and various signal transduction pathways. In fact, much of our understanding of sexual reproduction in filamentous fungi is based on similar pathways and processes that were first identified and studied in the yeasts (28). For example, the *MAT* genes and the pheromone genes were first described in *Saccharomyces cerevisiae* (29–31). Later, similar genes were identified in the genomes of many filamentous fungi (32–37) and, in some cases, these genes can fulfill their roles when heterologously expressed in *S. cerevisiae* (38, 39). This alone illustrates the phenomenal level of

conservation in the regulatory pathways of sexual development across diverse ascomycete fungi.

Despite the regulatory and genetic conservation of sexual reproduction in the yeasts and the filamentous fungi, there are notable differences, particularly with regard to the sexual tissues that are formed during the sexual cycle. In *S. cerevisiae*, compatible mating partners typically fuse and undergo karyogamy to form a diploid cell. Under conducive environmental conditions, this is followed by meiosis and the production of sexual progeny cells within a simple ascus as reviewed by Haber (40). In contrast, filamentous fungi form highly complex and multicellular sexual structures, including the ascomata (Fig. 1). Fungi residing in different orders produce ascomata of different shapes and sizes, but all of these structures contain asci in which the sexual ascospores are produced (reviewed in reference 41).

While ascomatal structures are morphologically diverse across the Pezizomycotina, the intermediate steps that result in the production of these tissues are relatively well conserved (Fig. 1). In many fungi, particularly self-sterile fungi, sexual reproduction begins with the fertilization of a female structure (ascogonium) by male structures, in the form of conidia or spermatia (42). Fertilization initiates the production of highly specialized cells and tissues, such as the protoascomata that mature into ascomata which bear asci. Also, within the asci, ascospores are produced via numerous rounds of meiosis and mitosis. The culmination of sexual development occurs when the ascospores are released into the environment to germinate.

The initiation of sexual reproduction, i.e., the step(s) before fertilization, differs from species to species. The requirements for initiation can be used to describe the four major sexual strategies found in the Pezizomycotina, i.e., heterothallism, primary homothallism, secondary homothallism, and unisexuality (Fig. 2). Individuals that exhibit heterothallic behavior are self-sterile, requiring a partner to engage in sexual reproduction (Fig. 1, step 1). Each of the mating partners carries a unique version of the *MAT* locus, and these must be different for sexual reproduction to occur. Each partner thus has a unique sexual identity, referred to as mating type, and this is conferred by the type and distribution of the *MAT* genes (Fig. 2).

It is worth noting that while the sexual and mating identities in heterothallic fungi can be described using mating type, these fungi also independently produce cells and structures that can be classified as male or female. Thus, in addition to being of the opposite mating type, compatible partners must also produce complementary male and female structures (43, 44). These two identities are not linked, and many heterothallic fungi are hermaphroditic, producing both male cells and female structures. Mating type is specifically determined by the genes present at the *MAT* locus, as discussed in detail below. In contrast, male and female structures are produced independently of the *MAT* information present. Importantly, a successful sexual interaction only requires the recognition of one set of male/female cells. Thus, male cells from an individual of the MAT1-1 type must interact with the female structures from an individual of the MAT1-2 type or *vice versa* (43, 44). Mating type and male/female identity primarily interact upon the expression of the mating pheromones, a process that is discussed in detail later in this review.

It is somewhat more difficult to distinguish between the different forms of homothallism at the physiological level because in all cases, a single isolate is capable of independent sexual development and is considered self-fertile (Fig. 2). These can, however, be differentiated genetically by studying the distribution of the *MAT* genes (Fig. 2) (45). In primary homothallism, all of the necessary *MAT* genes are present and expressed by a single individual and within a single nucleus (46). Secondary homothallism describes two different strategies: pseudohomothallism and mating type switching. In the former, the entire *MAT* complement is expressed by a single individual, but the *MAT* information is distributed between two independent nuclei that produce a heterokaryotic mycelium (47). Species capable of mating type switching harbor two versions of the mating information. One of these versions has a copy of all the *MAT*

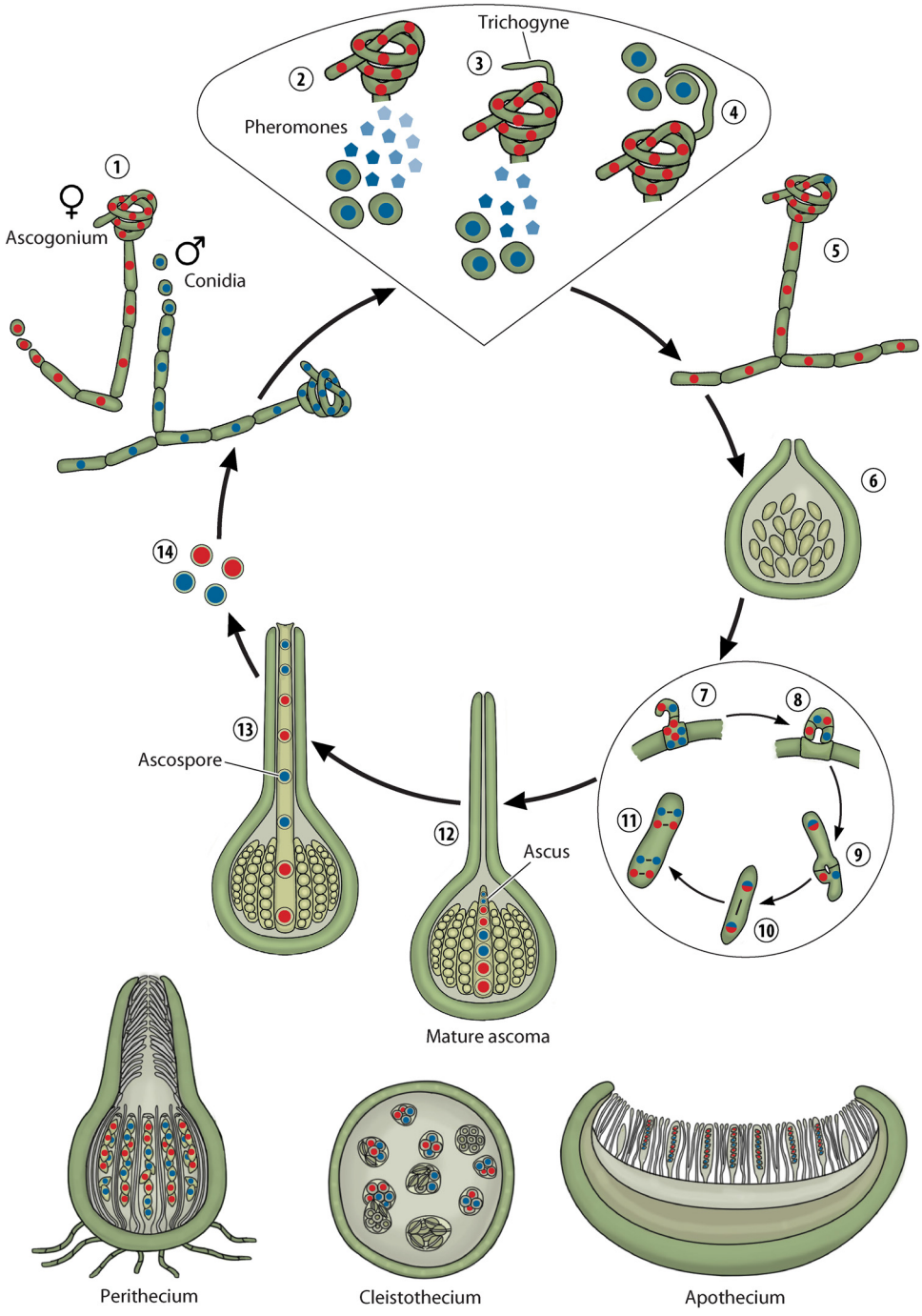

FIG 1 Generalized sexual cycle of heterothallic Pezizomycotina fungi. The cycle begins (step 1) with an interaction between compatible isolates, with male structures from one of the partners secreting pheromones (step 2), which are recognized by the female structures from a suitable mating partner (step 3). This recognition results in growth of the trichogyne toward the male cells (step 4), resulting in physical contact between the two partners; fertilization (step 5); and the subsequent production of a protoascoma (step 6). Within the ascus, multiple rounds of meiosis and mitosis produce the mature ascospores (steps 7 to 12), which are subsequently released (step 13) from the mature ascoma (step 14). This cycle would be identical in homothallic species, except that an opposite mating partner would not be required to initiate the process. The different ascomatal types are depicted below the sexual cycle, illustrating the morphological diversity that can be observed in the Pezizomyctonia.

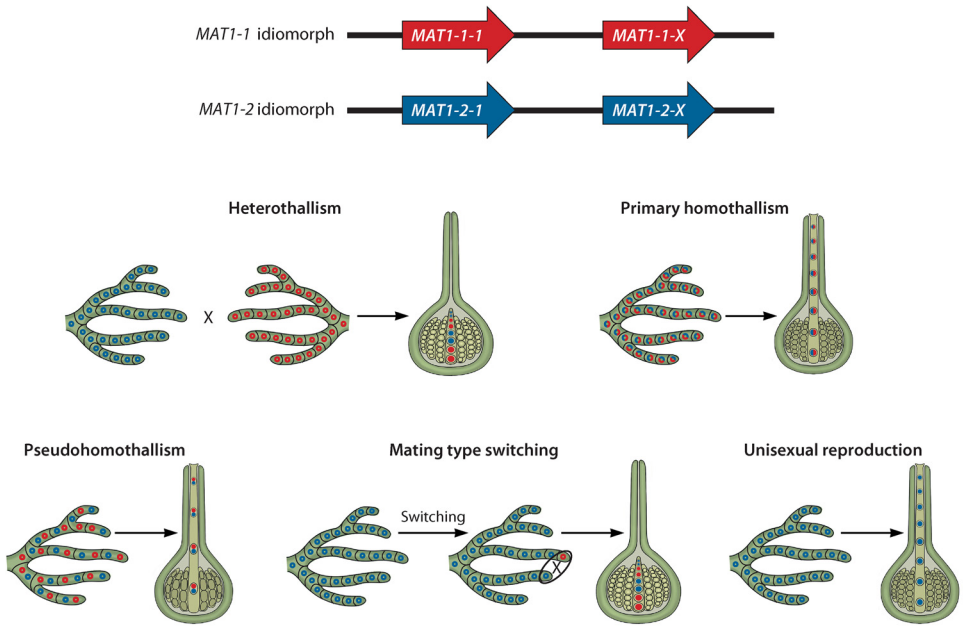

FIG 2 Mating-type idiomorphs, *MAT* genes, and their distribution in fungi utilizing the various sexual strategies exhibited by Pezizomycotina fungi. Open circles represent nuclei in each cell, while closed circles represent the ascospores produced. Red indicates the presence of genes from the *MAT1-1* idiomorph, and blue indicates the presence of genes from the *MAT1-2* idiomorph.

genes but can be altered to produce the second variant, which has a different gene complement (48, 49). Each of these two variants has a different mating specificity, and it is the expression of the *MAT* genes that drives compatibility between the two, thereby resulting in self-fertility in a single isolate (50). Unisexuality describes a system where a self-fertile individual retains and expresses only mating information from a single mating type (51).

The complex physiological processes underpinning sexual reproduction are stringently controlled by a huge number of genes. The primary regulators are encoded by the *MAT* genes, which regulate the expression of hundreds of sex-related genes (38, 52, 53), such as those involved in mating partner recognition. In addition, many non-*MAT*-regulated genes, including those involved in male and female fertility, ascomatal maturation and ascosporegenesis, are essential for the completion of the sexual cycle. The following sections provide details of the roles that each of these genes play.

MAT GENES

MAT1 Locus

The mating-type locus contains the *MAT* genes and has a primary role in the sexual process. This locus was formally described as *MAT1* based on the assumption that all ascomycetes would have a single copy of this region (54). Two versions of the *MAT1* locus are present in the diallelic mating system of heterothallic fungi, and this is recognized by naming these *MAT1-1* and *MAT1-2*, with the use of capital letters acknowledging the lack of dominance between the two "alleles" (Fig. 2) (54). The nonallelic nature of these versions was formally acknowledged in 1990, when the term idiomorph was suggested by John Wyatt and introduced by Metzenberg and Glass (55) to describe these two versions of the *MAT1* locus. Although the two different idiomorphs can easily be recognized in most heterothallic species, the term does not extend to their homothallic counterparts. Most homothallic species have a single *MAT1* locus that is defined based on the presence of homologous mating-type genes (56), although in some cases

more than one, often unlinked, *MAT* locus can be present within a single genome (32, 57, 58).

Mating-Type Genes

The precise definition of what constitutes a *MAT* gene is centered around their position in one of the two mating-type idiomorphs (56). This definition is broadly accepted, but an alternative description holds that a true mating-type gene is involved in establishing the mating-type identity of the nuclei after fertilization and that its protein product would localize only to the nucleus (59). Although such a definition may be considered the golden standard, it is impractical because many mating-type genes have not been the subject of functional studies. There is also convincing experimental and computational evidence that at least one of the known mating-type proteins, MAT1-1-2, localizes only to the cytoplasm and not the nucleus (60, 61). Furthermore, some homologous *MAT* genes have different functions in different species, precluding the application of function as an indication of mating identity. In the absence of a more suitable option, a system that defines mating-type genes as those present at the mating-type locus remains the most intuitive.

The locus-based definition for mating-type genes has been subject to valid criticism. Named *MAT* genes are widely considered to be the master regulators of sexual reproduction, responsible for conferring sexual identity and orchestrating processes linked to the sexual cycle. These roles can be validated only through functional studies (56, 62), which are lacking for most *MAT* genes. This implies that newly described *MAT* genes are mating-type genes in name only. This disparity is now being addressed, with new functional studies of diverse *MAT* genes being published regularly (63–65). Another criticism of a locus-based *MAT* gene definition is that the dynamic nature of this region can result in the inclusion of genes with no known role in mating into the *MAT1* locus (66, 67). This contradicts the position-based definition of a mating-type gene, a shortcoming highlighted in a recent revision of the *MAT* gene nomenclature system (56). Assigning a novel, *MAT*-specific name to a gene within the *MAT1* locus, only when no known homolog exists, can resolve this issue (56). This approach has been successfully applied to many novel *MAT* genes (68–71). In cases where homologs of known genes have been observed, these are significantly diverged to the point of representing *MAT*-specific versions of these genes (67, 72). Future studies must consider whether these genes have gained novel, *MAT*-specific functions.

Primary Mating-Type Genes

Two *MAT* genes are almost universally present in all studied Pezizomycotina classes (Table 1). *MAT1-1-1* was named as the first gene (*MAT1-1-**1***) from the *MAT1-1* (***MAT1-1***-*1*) idiomorph (73, 74), and its presence defines that idiomorph. Similarly, the *MAT1-2-1* gene typifies the *MAT1-2* idiomorph as the first described gene from this idiomorph (73, 75). The protein products of both these genes have conserved domains involved in DNA binding (76). In addition, a conserved intron is present in the *MAT1-2-1* HMG-box domain positioned across a serine codon, a characteristic that is unique to mating-type genes that have this domain (56, 77, 78). The core roles of *MAT1-1-1* and *MAT1-2-1* in the sexual process, as well as their wide distribution across the Pezizomycotina, has led to these genes being known as the core, or primary, mating-type genes (56, 62).

Secondary Mating-Type Genes

Apart from *MAT1-1-1* and *MAT1-2-1*, 11 additional *MAT1-1*-specific and 14 additional *MAT1-2*-specific genes have been described in the Pezizomycotina (56, 68, 70, 79, 80) and are all considered secondary *MAT* genes (Table 1). Although a formal definition for secondary mating-type genes does not exist, these genes are present within the confines of the mating-type locus, have no known homologs outside the mating-type genes, and tend to show a lineage-specific distribution (56). *MAT1-1-2* and *MAT1-1-3* have been identified from species in the Sordariomycetes and Leotiomycetes (56),

TABLE 1 *MAT* genes described from the Pezizomycotina

Gene	Representative taxa	Representative protein NCBI accession no.	Conserved protein domain	Reference(s)
Primary *MAT* genes				
MAT1-1-1	*Neurospora crassa*	ESA43845	PF04769–MATα1 HMG-box	74, 202
MAT1-2-1	*Neurospora crassa*	AAA33598	PF00505–HMG box	55, 75
Secondary *MAT* genes				
MAT1-1-2	*Neurospora crassa*	EAA35087	PF17043–MAT1-1-2 domain of unknown function	203
MAT1-1-3	*Neurospora crassa*	EAA35088	PF00505–HMG box	204
MAT1-1-4	*Pyrenopeziza brassicae*	CAA06845	None	204
MAT1-1-5	*Botrytis cinerea*	AHX22633	None	205
MAT1-1-6	*Pseudogymnoascus destructans*	AIG95729	None	206
MAT1-1-7	*Coccidioides immitis*	ABS19617	None	67
MAT1-1-8	*Diplodia sapinea*	AHA91691	None	207
MAT1-1-9[a]				
MAT1-1-10	*Morchella importuna*	MG681026	None	68
MAT1-1-11	*Morchella importuna*	MG681027	None	68
MAT1-1-12	*Teratosphaeria zuluensis*	QEJ80698	None	69
MAT1-2-2	*Neurospora crassa*	–[b]	None	85, 208
MAT1-2-3	*Coccidioides immitis*	KMP00264	PF11051–mannosyl trans3	67
MAT1-2-4	*Aspergillus fumigatus*	XP_754990	None	63
MAT1-2-5	*Diplodia sapinea*	AHA91682	None	207
MAT1-2-6	*Magnaporthe grisea*	BAE66607	PF00505–HMG box	209
MAT1-2-7	*Huntiella omanensis*	AOY41711	None	71
MAT1-2-8	*Villosiclava virens*	AKE48512	None	210
MAT1-2-9	*Fusarium fujikuroi*	AEP03799	None	211
MAT1-2-10	*Botrytis cinerea*	CDF43998	None	205
MAT1-2-11	*Pseudogymnoascus destructans*	AIG95713	None	206
MAT1-2-12	*Teratosphaeria zuluensis*	QEJ80704	None	69
MAT1-2-13	*Calonectria pauciramosa*	QKY89075	None	70
MAT1-2-14	*Letharia columbiana*	QEL51134	None	81
MAT1-2-15	*Colletotrichum fructicola*	XM_032032861	None	80

[a]A recent paper synonymized *MAT1-1-7* and *MAT1-1-9*, leaving the *MAT1-1-9* gene name vacant (81). See the text for more details.
[b]The *N. crassa MAT1-2-2* gene was predicted, but never annotated in the GenBank database. See reference 56 for detail.

while homologs of *MAT1-1-7* have been reported among the Lecanoromycetes and Eurotiomycetes (81), making these three genes the most widely spread secondary *MAT* genes. In a small number of Sordariomycetes, the *MAT1-1-2* gene is unusually present in the *MAT1-2* idiomorph (66, 82, 83), although this does not impact the sexual cycle in these fungi. Most other *MAT* genes have been identified in only a single order, family, or genus (56).

Since the last overview and revision of all known mating-type genes (56), four secondary *MAT1-1* genes have either had names assigned or amended. A homolog of the known *MAT1-1-7* gene was recently identified in several families of the Lecanoromycetes (81). Through positional conservation and phylogenetic analysis, it was shown that this gene is also homologous to the *MAT1-1-9* gene described from members of the Eurotiomycetes (81), leaving the *MAT1-1-9* gene name vacant (Table 1). *MAT1-1-10* and *MAT1-1-11* have been described from the *MAT* loci of *Morchella* species (Pezizomycetes) (68). Another putative *MAT1-1-10* gene was described in the Dothideomycete genus *Teratosphaeria* (69, 84) and appears also to be present in the *MAT1* locus of other members of the *Mycosphaerellaceae* (69). There is no apparent homology between the two *MAT1-1-10* genes, and they follow a lineage specific distribution.

Four novel genes have been identified from the *MAT1-2* mating-type idiomorph. Two of these have been named *MAT1-2-12*—one in *Teratosphaeria* species (69, 84) and the other in *Calonectria species* (70). There is no homology between the two *MAT1-2-12* genes or their encoded proteins (B. Wingfield, unpublished data). According to the guidelines of Wilken et al. (56), the *Teratosphaeria MAT1-1-10* (69, 84) should be renamed *MAT1-1-12*, while the *Calonectria MAT1-2-12* (70) should be known as *MAT1-2-*

13. A novel *MAT* gene was also described in species of the *Lecanoromycetes* and has been named *MAT1-2-14* (81), while another gene identified in *Colletotrichum* species was added as *MAT1-2-15* (80). None of these genes have any conserved domains that might suggest their possible functions.

The complexity in naming novel mating-type genes, together with the need to constantly reevaluate already established *MAT* gene names, highlights the need for a dynamic, curated database of mating-type gene names and homologs. A web-based *MAT* gene server to assist in mating-type gene identification is currently being developed (B. D. Wingfield, unpublished data) and will go some way to address these challenges.

Functions of Mating-Type Genes

Functional analyses remain the only true test for the role of a gene in the biology of a fungus, but such studies are lacking for most of the *MAT* genes. It is not surprising that the majority of available functional studies have targeted the core *MAT1-1-1* and *MAT1-2-1* genes, largely due to their wide distribution and central role in the sexual process (25, 52, 53, 60, 85–87). More recently, some studies have emerged that target the secondary *MAT* genes, illustrating the complex interactions that underlie sexual reproduction (63, 64, 85, 86, 88).

The history of functional studies on *MAT* genes has closely followed technological advances. Early studies relied on the transformation of cloned DNA into a complementary strain (33, 89). Later, mutations were induced in target genes by employing techniques such as the RIP mechanism that silences duplicate sequence products (25). The commercialization of molecular techniques relying on PCR amplification has strongly advanced new technologies used in functional studies. These include transcription profiling during sexual development (53, 87), comparisons of whole expression profiles between wild-type and mutant strains (53), antisense RNA approaches to disrupt gene function (87), and microarray experiments (52). Genome-based technologies have further expanded the available toolbox, with techniques such as CHIP-Seq analysis (90) and transcriptomics (91) becoming more prominent. The advent of novel, easy-to-use gene editing technologies, including the CRISPR-Cas9 system, promises further progress and has already led to the functional analysis of some mating-type genes (60, 64).

Functional studies on the *MAT1-1-1* and *MAT1-2-1* genes and their core domains are too numerous to cover in detail here. They have, however, provided an impression of conserved functions intermingled with great adaptability. Generally, both primary mating-type genes rarely impact vegetative growth directly (25, 52, 87, 88, 92), although they are necessary for successful completion of the sexual cycle in both homothallic and heterothallic fungi (25, 38, 52, 60, 75, 87, 92, 93). *MAT* genes control the sexual process by conferring sexual identity to individual isolates (34, 75), mediating the formation of male and female reproductive tissues (88, 93, 94), regulating the mating pheromone and receptor system (35, 52, 53, 88), and controlling ascomatal and ascospore development (34, 38, 88, 95). Although these functions appear to be well conserved, even among distantly related species, it is worth noting that these effects may not be universal. For example, in the smut fungus *Villosiclava virens* that causes false smut in rice, deletion of the *MAT1-1-1* gene has a significant impact on the vegetative growth rate and conidial structure (60). Whether this species is an exception or provides evidence of as-yet-undiscovered complexity in the functions of the mating genes remains to be discovered.

Among the secondary *MAT* genes, *MAT1-1-2* and *MAT1-1-3* have been the subject of most of the functional studies. Such studies have shown that these genes are generally not essential for sexual reproduction but rather function to fine-tune the sexual process. Early work on the model species *N. crassa* showed that both *MAT1-1-2* and *MAT1-1-3* influence the expression of sex-related genes, with mutants showing reduced fertility (25, 43). In contrast, in *Fusarium graminearum* both genes are important for male and female fertility, as well as ascospore formation (96). Once again, there are

exceptions to this generalization, such as the *Sordaria macrospora MAT1-1-2* gene, which is essential for completion of the sexual cycle (85). In some cases, this gene is also involved in functions attributed to the core *MAT* genes, including nuclear recognition, control of the pheromone response pathway, and ascomatal development (43, 59, 85). Deletion of the *S. macrospora MAT1-1-3* gene shows no observable phenotype (85), although deletion of the homologous gene in some homothallic *Fusarium* species abolishes self-fertility (97) and leads to significantly reduced fertility in *Podospora anserina* (43).

An interesting finding that has emerged from studies on *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*, and *MAT1-2-1* genes is that many nonmating functions are also under the control of these genes. These include general metabolic processes and energy production (52, 53), stress responses (60), the production of secondary metabolites (90), virulence (60), vegetative growth (64), and other diverse biological processes. This should not be surprising since all of these genes encode proteins that are characterized by conserved DNA-binding domains that are associated with transcription factors (56, 98). Therefore, it was plausible that these proteins would be involved in regulating a complex network of gene expression to accomplish the sexual process (99). In addition, sexual reproduction is an involved process that relies on specialized tissue differentiation in response to environmental and genetic cues (62). Unfortunately, many functional studies only evaluate the effect of *MAT* gene disruption on the sexual process and do not screen for pleiotropic effects. As new functional studies on the *MAT* genes are conducted, the effect of the *MAT* genes on nonmating functions will likely become more clear.

Apart from *MAT1-1-2* and *MAT1-1-3*, only five additional secondary *MAT* genes have been functionally characterized. Deletion of the *MAT1-1-5* gene in *Sclerotinia sclerotiorum* reduces the production of spermatia and completely blocks ascomatal development, halting the sexual process (88). The *MAT1-2-10* gene in the same species is involved in regulation of the cytoskeleton, with deletion mutants showing delayed ascomatal development and, when mature, produce aberrant numbers of ascospores (88). In the closely related species *Botrytis cinerea*, the roles of *MAT1-1-5* and *MAT1-2-10* are different, with both proteins jointly regulating the transition to karyogamy (92). The *MAT1-2-9* gene in *F. graminearum* appears not to be essential for sexual reproduction, but its transcription patterns do mirror those of the primary *MAT* genes (97). The *MAT1-2-4* gene in *A. fumigatus* is another essential regulator of sexual reproduction, but deletion of this gene did not allow for the elucidation of a specific function in sexual reproduction (63). A gene knockout study of the *Huntiella omanensis MAT1-2-7* gene showed that this gene is essential for maturation of the ascomata, a function shared with other secondary mating-type genes (64).

The mating-type genes are themselves subject to genetic regulation, mostly as a response to environmental cues. Studies on the model species *P. anserina* have revealed a network of HMG-box motif genes that regulate sexual development, some through direct transcriptional control of the mating-type genes and their downstream targets (99). Two genes, *ste11* and *hmg8* were identified as directly controlling *MAT* gene expression. A third gene, *pro1*, connects environmental cues, such as nutrient starvation, directly to *MAT* gene expression by activating *hmg8* (100). In the heterothallic species *Trichoderma reesei*, the effect of light on the sexual process is mediated by the *env1* and *vel1* genes through direct transcriptional regulation of *MAT1-2-1* (101, 102). Many other environmental response genes have been linked either directly or indirectly to *MAT* gene expression, and these have been extensively reviewed by Wilson et al. (62). Although these studies have provided some clarity, a thorough understanding of the genes that control *MAT* gene expression is still needed to fully appreciate the complexities of sexual reproduction in the Pezizomycotina.

Despite great progress having been made in recent times to identify and characterize mating-type genes, substantial gaps remain in our knowledge of this subject. For example, *MAT* genes have not been formally described in many classes of fungi, and

even in the classes with known mating-type genes, some orders lack representation (Fig. 3). It has been shown that variations in mating strategy can be present among even members of the same genus (58, 66), and this is often linked to interesting variations in the mating-type genes or *MAT1* locus structure (71, 82, 83). Examples exist of gene conversions (e.g., *MAT1-1-1* to *MAT1-2-7* [103]) and locus expansions (66, 67) that have helped to shape the *MAT1* region, but much remains unknown regarding the evolution and maintenance of this important region. Future studies on *MAT* gene function, as well as the description of the *MAT1* loci in underrepresented groups (Fig. 3), will contribute to addressing current knowledge gaps.

FEMALE AND MALE FERTILITY

Regardless of their mating type, most heterothallic fungi are capable of producing both male and female structures. Conidia or other spermatizing cells act as the male cells, which act by fertilizing the female structures known as ascogonia (Fig. 1) (104). Given that these structures are produced independently of one another, there are genes whose functions are exclusively linked to either male or female fertility. As a direct consequence, certain isolates can be female fertile and male sterile or *vice versa* (105). Isolates that are considered male sterile are typically unable to produce spermatizing agents or are incapable of fertilizing the ascogonia of the female partner. In contrast, isolates considered female sterile are either unable to form ascogonia or protoascomata or produce protoascomata that are unable to mature.

Some of the most important genes that influence male and female fertility are the mating pheromones and their cognate receptors, respectively (37, 106, 107). In general, the pheromones are expressed by the male cells and are recognized by their receptors, which are present on the cells of the female ascogonia (107). Understandably, deletion of the pheromone factors or receptors results in either a decrease or complete loss of fertility and the entire sexual pathway. Given the complexity and importance of this pathway in sexual development, a later section of this review is devoted entirely to the pheromone response pathway.

Very few genes have been associated with male fertility in the filamentous fungi. Instead, many of the genes that have been shown to contribute to the early steps of the sexual cycle predominantly regulate female fertility (108–111). Thus, apart from certain *MAT* genes (96) and the pheromone genes (109, 112), only the *mcm1* gene in *Magnaporthe oryzae* has thus far been described as being essential to male fertility (113). Encoding a MADS box transcription factor, this gene is also required for the production of microconidia, which likely act as the spermatizing agents in this fungus. It is thus not surprising that Δ*mcm1* mutants are male sterile (113).

In *P. anserina*, four genes have been shown to contribute significantly to female fertility; *nox1* is important for the production of maternal tissues (114), *mpk1* is important for the development of the underlying mycelium that surrounds and nourishes the growing ascomata (115), and the two IDC genes, *idc2* and *idc3*, are involved in the communication between these two tissue types (116). It is thought that *nox1*, which encodes an NADPH oxidase, is responsible for providing the developing protoascomata with fresh nutrients via reactive oxygen species (ROS)-mediated signaling, and its deletion results in very small protoascomata and delayed development (114). Similarly, deletion of either of the IDC genes results in isolates that can differentiate into both male and female structures but exhibit delayed protoascomatal maturation after fertilization. In these isolates, some ascomata will develop, but only a few ascospores are released (116).

In *N. crassa*, three genes coding for proteins involved in the calcium ion signaling pathway are important for female fertility (117): a phospholipase (*plc1*), a secretory phospholipase (*splA2*), and a calcium-hydrogen ion exchanger (*cpe1*). Two double mutants, Δ*plc1*/Δ*splA2* and Δ*plc1*/Δ*cpe1*, are entirely female sterile and produce no ascomata. It is thought that these phenotypes are the result of a significant decrease in the expression of the two mating pheromones, as well as a female/male fertility gene

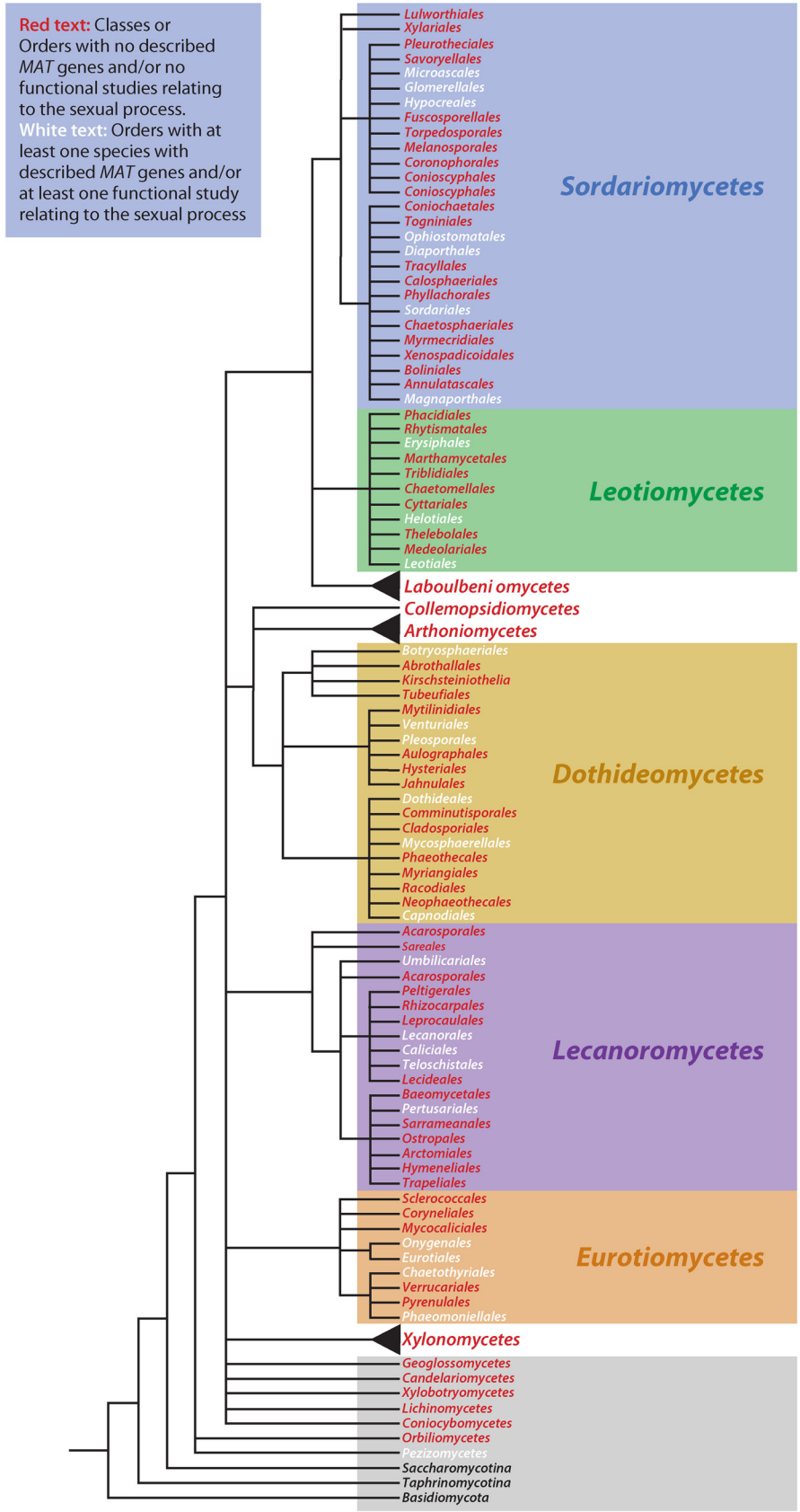

FIG 3 Cladogram of the Pezizomycotina highlighting the lack of studies on sexual reproduction in this group. Classes or orders shown in red have no described *MAT* genes and/or no published

(Continued on next page)

(*fmf-1*) in the double mutants (117). A fourth gene, *fig1*, is involved in a calcium ion uptake system and is essential for female fertility in MAT1-2 isolates of *N. crassa* (118), illustrating not only that calcium uptake and signaling are important for fertility, but that the role of a protein can change based on the mating type of the isolate.

RECOGNITION OF A SUITABLE MATING PARTNER

The sexual cycle, particularly in heterothallic species, begins with the identification and recognition of an appropriate mating partner (28). This process is facilitated in many species by the pheromone response pathway (7). Partners of opposite mating type express one of the two mating pheromones, which are secreted into the environment by male cells, and are subsequently recognized by a suitable opposite mating type partner via pheromone receptors (Fig. 1). Upon recognition of these diffusible peptides, specialized hyphae known as trichogynes grow from the female partner toward the male cell. Once the male and female cells interact physically, fertilization takes place, and the sexual cycle begins. At a molecular level, the pheromone factor/receptor recognition activates a heterotrimeric G-protein, thereby initiating the signal transduction pathway, which culminates in the transcription of genes related to mating (Fig. 4).

Mating Pheromones

The two most commonly utilized fungal mating pheromones are the α- and **a**-factors (7, 119). The genes encoding these small proteins are transcriptionally regulated by the MAT1-1-1 and MAT1-2-1 proteins, respectively. Consequently, the pheromone genes are expressed in a mating type-dependent manner in most heterothallic species, allowing for recognition between individuals of opposite mating type (35, 91, 120, 121).

Genes encoding the two pheromones have been described in species with different sexual strategies. These genes have been found in diverse heterothallic species such as *N. crassa* (35), *H. omanensis* (91), *C. parasitica* (120), and *Magnaporthe grisea* (121). Similarly, the homothallic species *F. graminearum* (122), *A. nidulans* (36), and *S. macrospora* (123, 124) all harbor genes encoding mating pheromones. Even species exhibiting the more unique strategies, such as unisexuality (*Huntiella moniliformis* [69] and *Neurospora africana* [125]) and pseudohomothallism (*P. anserina* [37]) express mating pheromones.

Interestingly, there are groups of fungi where only a single mating pheromone has been identified. This is despite a wealth of genomic and transcriptomic data and a significant effort to identify these genes. In *A. nidulans* (36) and other Eurotiomycete fungi (125), for example, the gene encoding a protein with similarity to the **a**-factor pheromone has remained elusive, while the α-factor pheromone has been identified. Similarly, species in the Pezizomycetes, including *Tuber melanosporum* (126), also harbor only the α-factor encoding gene. Whether a second factor exists, with or without similarity to the **a**-factor, remains to be discovered. These genes, being highly variable and typically very short, are notoriously difficult to identify. Their identification consequently requires gene sequences from very close relatives to be used as BLAST queries against the genomes of newly investigated species. Alternatively, when such sequences are not available, it is possible to make use of the microsynteny that exists between more distantly related species (125). In this way, pheromone-flanking genes in species where the pheromones are known can be used to identify the locus harboring the pheromone, after which the pheromone genes can be manually annotated. In addition, the conserved domains of the pheromones, as detailed below, can also be

FIG 3 Legend (Continued)
functional studies relating to the sexual process. Orders shown in white have at least one species with described *MAT* genes and/or at least one functional study related to sexual reproduction. Details can be found in Table S1 in the supplemental material. The cladogram was constructed in phyloT using NCBI taxonomic identification numbers and was edited in iTol v6 (https://itol.embl.de/).

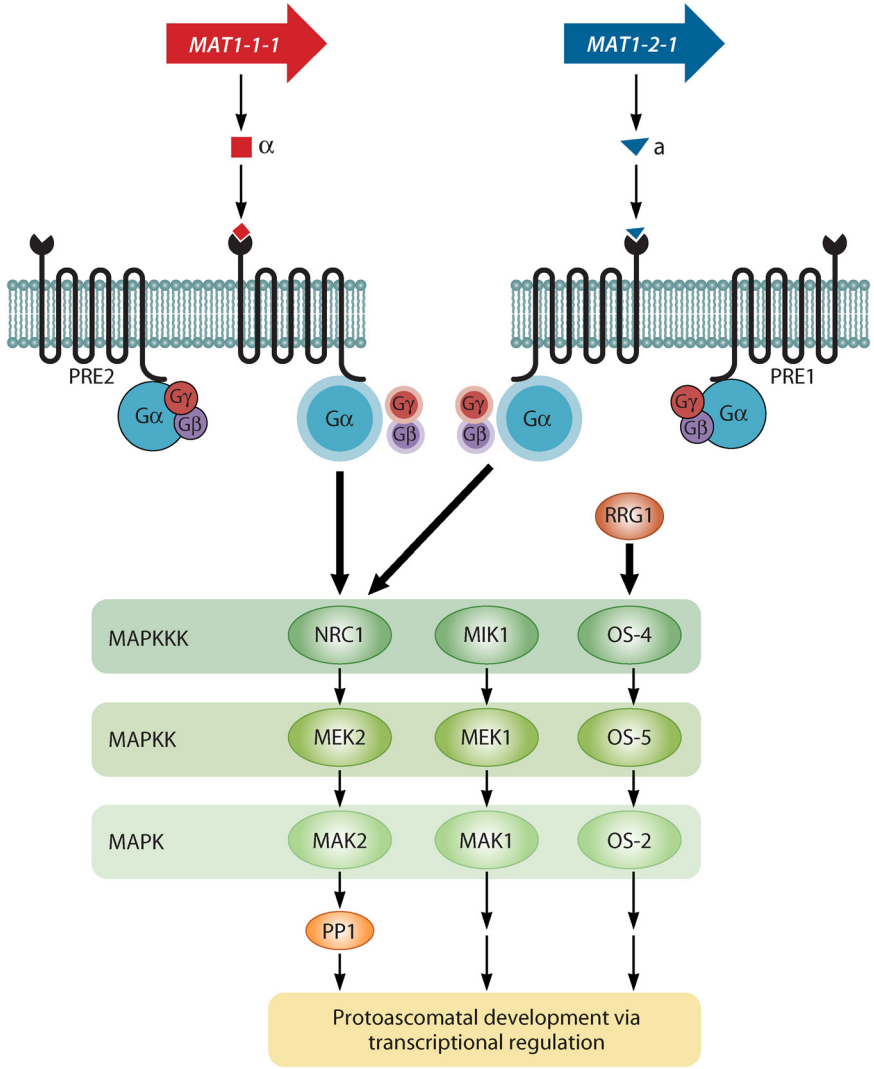

FIG 4 Pheromone response pathway in *Neurospora crassa*. The MAT1-1-1 and MAT1-2-1 proteins control the expression of the α- and **a**-factor pheromones, respectively. Recognition of the pheromones by their respective cognate receptors results in activation of the coupled G-protein and dissociation of its subunits. These subunits subsequently activate one of the three MAPK cascades, MAK2, that ultimately results in protoascomatal development. Two other MAPK cascades, MAK1 and OS-2, are also important for protoascomatal development but respond to different environmental signals.

used to search through and filter protein-coding genes in these genomes. This makes it possible to identify proteins that share structural similarities to the mating pheromones.

Given their important role in partner recognition, it is not surprising that the pheromone genes are essential for sexual reproduction in many model heterothallic species (Table 2). In *N. crassa* (106) and *C. parasitica* (112, 127), both pheromone types are indispensable for male fertility, with deletion mutants unable to complete the sexual cycle. In *N. crassa*, the importance of each pheromone in male fertility is mating type dependent (106). Thus, **a**-factor deletion inhibits sexual development in a MAT1-2 strain, while α-factor deletion precludes sexual development in a MAT1-1 strain. In addition, the **a**-factor pheromone also plays an important role in female fertility in *N. crassa* (109), a role that is not restricted to either mating type. Similarly, the α-factor is important for male fertility (112) in *C. parasitica*, while the **a**-factor pheromone is essential for female fertility (120).

TABLE 2 Comparison between the functions of proteins involved in the pheromone response pathway and MAPK cascades in heterothallic and homothallic species

	Neurospora crassa (heterothallic)		*Sordaria macrospora* (homothallic)			
Protein	**Gene**	**Mutant phenotypes[a]**	**Gene**	**Mutant phenotypes[b]**		
α-Factor pheromone	*ccg4*	Male sterility* in MAT1-1 partner (106)	*ppg1*	No sexual defects (128)	Δ*ppg1*/Δ*ppg2*: reduced ascomatal production (128)	Δ*ppg1*/Δ*pre1*: greatly reduced ascomatal production (128)
a-Factor pheromone	*mfa-1*	Male sterility in MAT1-2 partner (106); aberrant female development (108)	*ppg2*	No sexual defects (128)		Δ*ppg2*/Δ*pre2*: greatly reduced ascomatal production (128)
α-Factor receptor	*pre2*	No protoascomatal maturation in MAT1-2 partner (24)	*pre2*	No sexual defects (128)	Δ*pre1*/Δ*pre2*: no protoascomatal maturation (128)	Δ*ppg2*/Δ*pre2*: greatly reduced ascomatal production (128)
a-Factor receptor	*pre1*	No protoascomatal maturation in MAT1-1 partner (107)	*pre1*	No sexual defects (128)		Δ*ppg1*/Δ*pre1*: greatly reduced ascomatal production (128)
Gα subunit	*gna1*	Unusually shaped protoascomata that do not grow towards spermatia (150)	*gsa1*	Delayed and reduced ascomatal production (152)	Δ*gsa1*/Δ*gsa2* and Δ*gsa1*/Δ*gsa3*: greatly reduced protoascomatal production and no protoascomatal maturation (152)	
	gna2	No sexual defects (151)	*gsa2*	No sexual defects (152)		
			gsa3	Greatly reduced ascospore germination (152)		
Gβ subunit	*gnb1*	No protoascomatal maturation (111, 153)				
Gγ subunit	*gng1*	No protoascomatal maturation† (153)				
MAPKKK	*nrc1*	No protoascomatal production‡ (212)	*mik2*	No protoascomatal maturation (160)		
MAPKK			*mek2*	No protoascomatal maturation (160)		
MAPK	*mak2*	No protoascomatal production (155)	*mak2*	No protoascomatal maturation (160)		
Transcriptional regulator	*pp-1*	No protoascomatal production (155)	ste12	Reduced asci production, fragile walls of asci and ascospores (193)		
MAPKKK	*mik1*	No protoascomatal production (110)	*mik1*	No protoascomatal maturation (159)		
MAPKK	*mek1*	No protoascomatal production (110)	*mek1*	No protoascomatal maturation (159)		
MAPK	*mak1*	No protoascomatal production (110)	*mak1*	No protoascomatal maturation (159)		
	Rrg-1	No protoascomatal production (156)				
MAPKKK	*os-4*	No protoascomatal production (157)				
MAPKK	*os-5*	No protoascomatal production (157)				
MAPK	*os-2*	No protoascomatal production (156)				
Adenylyl cyclase	*cr-1*	Delayed ascomatal development (213)	*sac1*	Reduced ascomatal size and ascospore germination (152)		

[a]*, Male sterility indicates that an isolate is unable to act as the male partners; †, no protoascomatal maturation indicates that protoascomata are produced but do not mature into ascomata; ‡, no protoascomatal production indicates that protoascomata are not formed at all.

[b]Wild-type (proto)ascomatal production > reduced (proto)ascomatal production > greatly reduced (proto)ascomatal production.

The mating pheromones play an important role in the sexual development of the homothallic species, *F. graminearum* and *S. macrospora* (Table 2) (122, 128). While not essential for this pathway, gene deletion mutants in both species display decreased fertility, producing significantly fewer mature ascomata than wild-type isolates. This phenotype is observed in *F. graminearum* when the α-factor gene or both the α- and **a**-factor genes are deleted (122). In contrast, only the deletion of both pheromone genes induces this phenotype in *S. macrospora* (128). In both species, deletion of only the **a**-factor pheromone results in fully self-fertile isolates (122, 128), suggesting that this gene is not important in homothallic mating.

The strict association of the α-factor with the MAT1-1-1 protein and the **a**-factor with the MAT1-2-1 protein has some interesting implications and may play an important role in establishing the sexual strategies observed in various fungal species. This association is typically strongest in heterothallic species, where the pheromones are used to communicate the mating type of an individual to potential mating partners, thereby facilitating partner recognition (35, 91, 120, 121). This association is less stringent in homothallic species, with both the primary and secondary MAT proteins regulating pheromone expression. In *S. macrospora*, MAT1-1-1 positively regulates the expression of both pheromones, MAT1-1-2 negatively regulates **a**-factor expression while MAT1-2-1 positively regulates **a**-factor expression (52, 85). Similarly, in the homothallic *F. graminearum*, the MAT1-1-1, MAT1-1-2, MAT1-1-3, and MAT1-2-1 proteins all negatively regulate the expression of both pheromone factors to various degrees (96). The lack of a requirement to attract and recognize partners of opposite mating type has evidently changed the *MAT*-dependent regulation of the pheromones, even while these mating factors remain important for sexual development in these species.

Pheromone expression and regulation is particularly noteworthy in species with the more unique mating strategies. Both pheromones are expressed in the two unisexual species, *H. moniliformis* and *N. africana*, despite the absence of *MAT1-1*-associated genes in *H. moniliformis* and *MAT1-2*-associated genes in *N. africana* (91, 125). Similarly, species of the genus *Colletotrichum* are known to exhibit various mating strategies, including mating type switching (129) and unbalanced heterothallism, a type of self-sterility in which suitable mating partners have complementary mutations in genes involved in sexual reproduction (130). This is despite the fact that these species harbor only genes associated with the *MAT1-2* idiomorph (80). These species have also been shown to have undergone concerted gene loss events associated with both pheromone factors, with some species harboring only one of the two pheromones and some species harboring neither (80). This suggests that the pheromones in these species may be regulated in a *MAT*-independent manner that may, at least partly, explain their divergence from the typical mating strategies.

Fungal mating pheromones are produced as larger preproteins that are subsequently processed into smaller, mature pheromone factors that are functionally active (Fig. 5) (7). Much of our understanding regarding the processing of mating pheromones in fungi arises from research conducted on *S. cerevisiae* (7). However, many of the processing proteins found in this model yeast have homologs in filamentous fungi and are thought to play similar roles in pheromone processing.

Processing of the a-factor. The defining feature of the **a**-factor preprotein is a terminal CaaX domain, a region comprised of a cysteine residue followed by two aliphatic residues and an amino acid of any type (Fig. 5, left) (131). This highly conserved domain is posttranslationally modified by a variety of other proteins before the mature pheromone factor is secreted (reviewed in reference 7). The first posttranslational modification occurs when RAM1 and RAM2 proteins farnesylate the cysteine residue of the CaaX domain. The three C-terminal residues are then removed by RCE1 or STE24, where after STE14 adds a carboxymethyl group to the now-terminal C residue. The final step is the proteolysis of the N-terminal region by STE24 and AXL1. This produces a mature factor which is subsequently secreted by the STE6 transport protein.

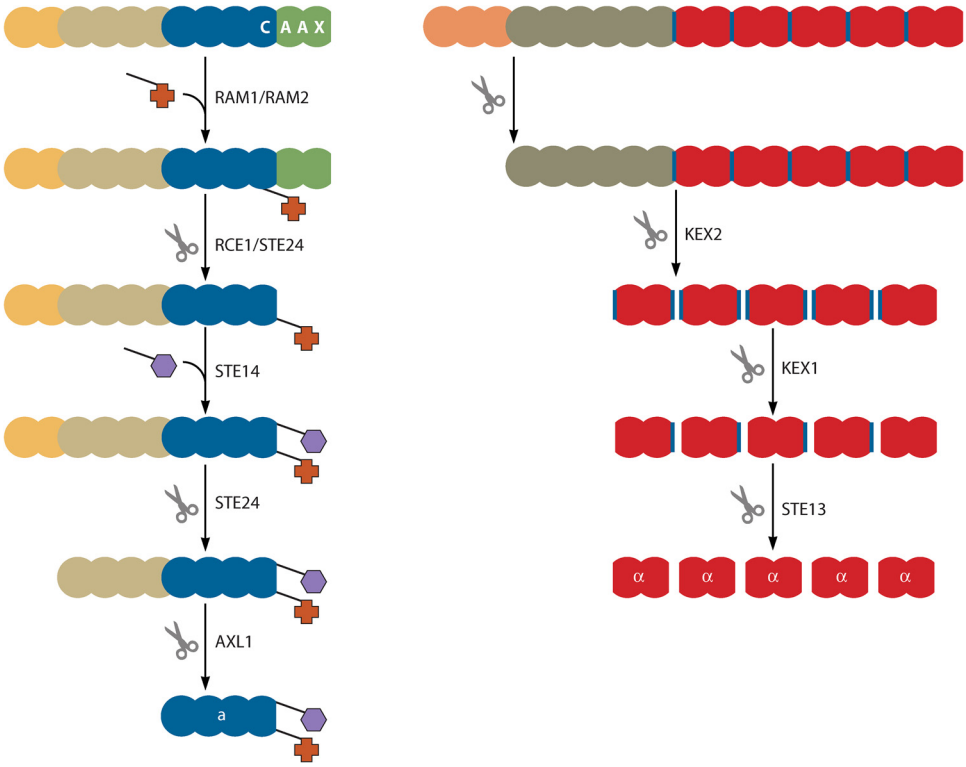

FIG 5 Structure and processing of **a**- and α-factor mating pheromones. Both fungal mating pheromones are initially expressed as large preproteins which are processed into small, mature pheromone factors. The first step in the processing of the **a**-factor includes the addition of a farnesyl (indicated by the "**+**" symbol) by RAM1/RAM2. This is followed by cleavage of the terminal AAX amino acids by RCE1/STE24 and the addition of a carboxymethyl group (indicated by a hexagon symbol) by STE14. Two final cleavage events, facilitated by STE24 and AXL1, release the mature pheromone. The first step in α-factor processing is the removal of the signal peptide (indicated in dark orange). KEX2-, KEX1-, and STE13-mediated cleavage releases numerous copies of the mature pheromone.

The **a**-factor pheromone processing pathway has not been elucidated in any filamentous fungi, but many of these fungi contain genes encoding homologs of the *S. cerevisiae* processing proteins. Almost all of these genes have been identified in the genomes of species as diverse as *A. fumigatus* (132), *A. cristatus* (133), *A. niger* (134), *M. oryzae* (135), *N. crassa* (109), *Penicillium marneffei* (141), *P. anserina* (53), and *Verticillium dahliae* (136). An interesting exception is *axl1*, which has only been described in *N. crassa* (109). The fact that most of these processing proteins, as well as the **a**-factor pheromone, have all been maintained in the Pezizomycotina strongly implies that there may be some conservation in the entire processing pathway.

Unfortunately, very little research has been conducted on the function of the pheromone processing genes in the Pezizomycotina. Consequently, it remains unclear whether they play the same roles in pheromone processing as they do in *S. cerevisiae*. Experimental evidence does suggest that the two RAM proteins play a far more general role in the biology of these fungi than simply being involved in pheromone processing. The deletion of *ram1* and *ram2* is lethal in *P. anserina* (53) and *M. oryzae* (135), respectively, whereas *ram1* disruption in *M. oryzae* affects processes as diverse as growth, stress resistance, and virulence (135). This is perhaps not surprising because the RAM proteins are posttranslational modifiers that add farnesyl groups to proteins in order to ensure their appropriate cellular localization (137). This form of modification affects a large number of proteins, particularly those involved in signal transduction, and consequently affects a variety of biological processes not limited to mating (135).

Manipulation of the *A. cristatus MAT1-1-1* and *MAT1-2-1* genes provides further experimental evidence to support the importance of the **a**-factor pheromone processing

proteins in the sexual development of filamentous fungi. The *rce1* and *ste14* genes, both involved in some of the final processing steps, are significantly downregulated in *MAT1-1-1* and *MAT1-2-1* mutants, suggesting that these genes are (in)directly under the control of these two master regulators (133). Similarly, under conditions of high osmolarity, where sexual reproduction is precluded, *rce1* is downregulated in *A. cristatus* (138). While such results do not confirm a function for these proteins in pheromone processing, they certainly imply a role for these genes during sexual development.

Processing of the α-factor. The α-factor preprotein harbors a hydrophobic N-terminal secretion signal, a proregion and repeating units of the mature pheromone sequence (Fig. 5, right) (reviewed in reference 7). The first step in processing is the removal of the secretion signal by a signal peptidase in the endoplasmic reticulum. This is followed by proteolytic cleavage and removal of the proregion by the KEX2 protein. Once the N terminus has been removed, the preprotein is left containing repeats of a mature α-factor. Release of the mature pheromone peptides relies on the presence of two dipeptide motifs that flank these repeats. The first, either the dipeptide KR or RR, is a KEX2-like peptidase recognition site. KEX2 activity releases the numerous immature pheromone peptides, which are then subjected to terminal amino acid removal by KEX1. The final step in the processing of this pheromone is due to STE13-like aminopeptidase activity, which recognizes the second dipeptide motif, either XA or XP. Finally, STE13 activity releases the mature pheromone, which is subsequently packaged into vesicles and secreted via the classical secretion pathway.

Genes encoding the KEX1, KEX2, and STE13 processing proteins have been found in the genomes of various filamentous fungi, including *S. macrospora* (39, 139), various *Trichoderma* species (140), *A. fumigatus* (132), *A. nidulans* (32), *N. crassa* (35), *P. marneffei* (141), *H. omanensis* (91), and *H. moniliformis* (91). Similarly, the processing sites for these proteins are present in the α-factor pheromone preproteins of these species, suggesting conservation in the processing of these proteins across yeast and filamentous fungi. This has been experimentally proven in *S. macrospora*, where the α-factor preprotein in this species can be properly processed, secreted, and is functional in *S. cerevisiae MAT*α cells (39).

Gene deletion and silencing studies in *N. crassa* and *C. parasitica* have further supported the role of these processing proteins in the sexual development of filamentous fungi. Fertility in *N. crassa* is influenced by KEX1, where deletion of the *kex1* homolog, *scp*, results in greatly reduced male fertility (35, 142). Unsurprisingly, this effect is only apparent in the α-factor-expressing MAT1-1 cells, while MAT1-2 cells are unaffected by the gene's deletion. Similarly, *kex2*-silencing in *C. parasitica* also results in reduced male fertility and an inability to produce ascospore-bearing ascomata (143). Lastly, a genome-wide gene expression study in *N. crassa* also showed that the expression patterns of *ste13* closely mimic those of the α-factor pheromone, both increasing during ascomatal development (144). This hints at a role for this protein in the sexual cycle.

Cognate Receptors

Each of the mating pheromones is recognized by a specific cognate receptor. Both receptors are seven-transmembrane domain proteins, which are comprised of an extracellular recognition domain, seven membrane spanning domains and an intracellular domain that is linked to a G-protein (Fig. 4) (reviewed in references 145 and 146). The **a**-factor pheromone is recognized by the PRE1 protein, which has similarity to the STE3 protein from *S. cerevisiae* (107). Similarly, the PRE2 protein, which is similar to the *S. cerevisiae* STE2 protein, is the α-factor's cognate receptor (38).

As with the pheromone factors themselves, the pheromone receptor genes have been identified and functionally characterized in many filamentous ascomycete fungi (24, 53, 107, 122, 128). While these genes are found in both heterothallic and homothallic fungi, their relative importance in sexual development differs from species to species (Table 2). In some species, both of the receptor genes are essential for sexual development, while in others these genes are dispensable, although their deletion may result in decreased fertility (53, 128, 147, 148).

An interesting characteristic of the two pheromone receptor proteins is that they often have mating type-dependent roles in heterothallic species. In these species, the PRE1 protein plays an important role in female fertility in MAT1-1 isolates, while female fertility in the MAT1-2 isolates depends on the PRE2 protein. This is understandable given that MAT1-1 isolates express the α-factor and need to recognize the **a**-factor via the expression of *pre1*, while the converse is true of the MAT1-2 isolates. This mating type-dependent effect has been observed in *P. anserina* (53), *N. crassa* (24, 107), and *T. reesei* (147), where Δ*pre1* MAT1-1 and Δ*pre2* MAT1-2 isolates are female sterile-producing protoascomata that are incapable of being fertilized and thus do not mature into ascospore-bearing ascomata. Importantly, Δ*pre1* MAT1-2 and Δ*pre2* MAT1-1 isolates interact normally with wild-type partners and produce fully mature ascomata and viable ascospores.

The pheromone receptors are important, but typically not essential, for the sexual cycle in a number of homothallic species (Table 2). In both *S. macrospora* (128) and *A. nidulans* (148), the deletion of both *pre1* and *pre2* results in the complete loss of the sexual cycle by precluding the production of ascomata. However, deletion of a single receptor produces no detectable effect in *S. macrospora* (128) but does result in reduced fertility in *A. nidulans*, with mutants producing smaller ascomata and fewer ascospores (148). In *F. graminearum*, *pre1* is dispensable for sexual development, with Δ*pre1* isolates sporulating normally (122). In contrast, single Δ*pre2* mutants and double Δ*pre1*/Δ*pre2* mutants exhibit subnormal fertility levels (122).

Signal Transduction Pathways

As G-protein coupled receptors (GPCRs), the pheromone receptors are linked to a G-protein via their terminal intracellular domains (Fig. 4) (149). Prior to pheromone recognition, the G-protein exists as a trimer of three protein subunits: Gα, Gβ, and Gγ. Once the pheromone has physically bound to the receptor, the G-protein is activated, resulting in dissociation of the subunits into a Gα monomer and a Gβ-Gγ dimer, each of which is capable of initiating a downstream signal transduction cascade (149).

Given their close association with the pheromone receptors, it is not surprising that all three subunits play an important role in female fertility (Table 2). Two genes, *gna1* (150) and *gna2* (151), encode Gα-subunits that have been functionally characterized with respect to their roles in mating in the heterothallic *N. crassa*. Deletion of *gna1* results in mutant isolates that produce unusually shaped protoascomata (150) and are incapable of growth toward the spermatia (84). There is consequently little or no maturation of these structures into mature ascomata. Interestingly, *gna2* deletion mutants do not produce particularly notable sexual defects, but Δ*gna1*/Δ*gna2* mutants are more severely affected than single *gna1* deletion mutants (151). This fact illustrates that while the two α-subunits may have overlapping functions, the GNA1 protein can compensate for GNA2 but not *vice versa*. Deletion of the Gα-subunit gene in *S. macrospora*, results in a similar phenotype; though, while protoascomatal production is delayed and reduced in Δ*gsa1* mutants, mature ascomata are produced after sufficient incubation (152). This could reflect a change in the importance of the pheromone response pathway in the homothallic *S. macrospora* compared to the heterothallic *N. crassa* (Table 2).

The *N. crassa* β- and γ-subunits, encoded by *gnb1* and *gng1*, respectively, are essential for the maturation of normal ascomata. Single and double mutants of the *gnb1* and *gng1* genes are all female sterile, producing immature protoascomata that do not develop further, even when adequately fertilized (111, 153). On the rare occasion where mature ascomata are produced, they remain small and are unable to eject the ascospores (111). It is perhaps not surprising that deletion of the Gα-, Gβ-, and Gγ-subunits produce similar phenotypes and, in fact, the expressions of the three subunit genes are controlled by one another. For example, *gnb1* deletion results in the complete downregulation of *gna1* and *gng1* expression (111, 153), whereas *gng1* deletion results in significant downregulation of *gnb1* (153).

After dissociation, the Gα and Gβ-Gγ subunits often act by activating mitogen-activated protein (MAP) kinase signal transduction pathways that are capable of eliciting a

variety of effects, including the initiation of sexual reproduction via the activation or repression of relevant transcription factors (Fig. 4) (reviewed in reference 154). In general, the first component of this kind of transduction pathway is a MAP kinase kinase kinase (MAPKKK), followed by a MAPKK, a terminal MAPK and, finally, the targeting protein, which is often a transcription factor. In *N. crassa*, three of these pathways are involved in sexual development and are named after their terminal kinases, i.e.,. MAK1 (110), MAK2 (155), and OS2 (156, 157) (Fig. 4). Intriguingly, while all three pathways have been implicated in protoascomatal development, only the MAK2 pathway is directly linked to the mating pheromone receptors (Fig. 4) (158). This suggests that the cross talk between these three pathways is essential for sexual development in *N. crassa*. Thus, the deletion of even a single gene in one of these pathways results in female sterility (Table 2) (110). The MAK1 (159) and MAK2 (160) pathways are also important for sexual reproduction in *S. macrospora*, where single gene deletions also preclude sexual development (Table 2).

There are some important differences between heterothallic and homothallic fungi relating to the pheromone response pathway and various MAPK cascades. To illustrate these differences, the mutant phenotypes of genes involved in the heterothallic *N. crassa* and homothallic *S. macrospora* are shown in Table 2. What is evident is that the proteins that play a role during the initiation of the pheromone response pathway, i.e., the pheromone factors, their receptors, and the coupled G-proteins, are less important for sexual development in *S. macrospora* than they are in *N. crassa*. In fact, single gene deletions in *S. macrospora* either result in no sexual defects or simply delay sexual development. In contrast, similar deletions in *N. crassa* drastically affect male and/or female fertility. When the MAPK cascades are considered, however, the effects of single gene deletions in the heterothallic and homothallic species are comparable. This suggests that while the process of initiating sexual recognition is different in heterothallic and homothallic fungi, the downstream processes are the same.

PROTOASCOMATAL DEVELOPMENT AND ASCOMATAL MATURATION

After partner recognition occurs, male cells are capable of fertilizing the female ascogonia, which will begin to develop into a protoascoma [reviewed in reference (41)]. It is within this immature sexual structure that the various stages of nucleus migration, karyogamy, mitosis and meiosis will commence. During this time, the protoascoma develops into a mature ascoma containing asci and the sexual ascospores. Three protein complexes and/or pathways (the STRIPAK complex, the PACC pathway and the COP9 signalosome) have been specifically linked to ascomatal development in the filamentous fungi (Fig. 6).

STRIPAK Complex

One of the most important protein complexes that controls the development of the ascoma is the striatin-interacting phosphatases and kinases (STRIPAK) complex (Fig. 6) (161, 162). In fungi, this complex is made up of a protein phosphatase (PP2AA), a striatin (PRO11), a striatin-interacting protein (PRO22), a phocein (MOB3), kinases (KIN3, KIN24), a membrane-anchoring protein (PRO45), and many other proteins (162). This complex is highly conserved across the eukaryotes and is thought to be involved in various signal transduction pathways important for the differentiation of multicellular structures (161).

The first fungal STRIPAK subunits were identified in *N. crassa* (163), and some research has been conducted to elucidate the roles that these proteins play in this species (163–165). However, this complex, its functions, and the protein-protein interactions are much better understood in *S. macrospora* (reviewed in reference 162). The *S. macrospora* STRIPAK complex appears to be involved in diverse biological processes due to its primary function being the (de)phosphorylation of a variety of target proteins (162). Many of these targets are known to play important roles in sexual development (166). For example, the α-factor pheromone processing proteins KEX1 and KEX2 are both (de)phosphorylation targets of the STRIPAK complex in *S. macrospora*.

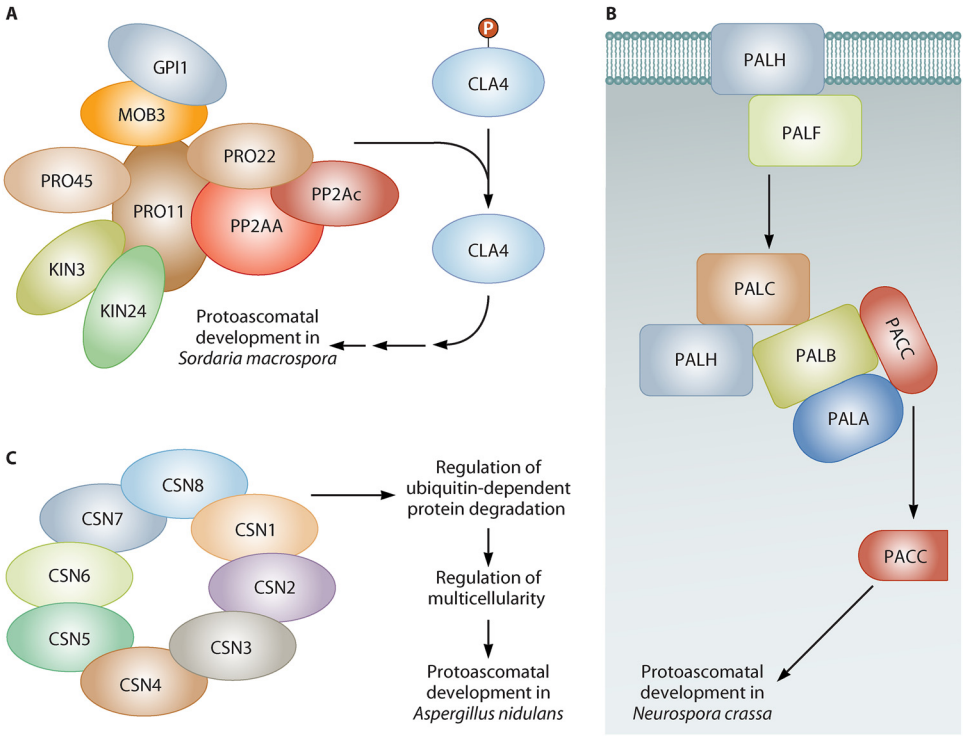

FIG 6 Important protein complexes and pathways involved in protoascomatal maturation and ascomatal development in various filamentous fungi. (A) The STRIPAK is important for sexual development in the homothallic *Sordaria macrospora*. (Based on data from reference 161.) (B) The PACC pathway plays an essential role in protoascomatal development in the heterothallic *Neurospora crassa*. (Adapted from reference 175.) (C) The COP9 signalosome is important for sexual development and other developmental processes in the homothallic *Aspergillus nidulans*. (Based on data from references 166 and 186.) Protein names and/or abbreviations: GPI1 (glycosylphosphatidylinositol-anchored protein); MOB3 (monopolar spindle one-binder protein); PP2Ac and PP2Aa (protein phosphatase subunits); KIN3 and KIN24 (kinases 3 and 24); CLA4 (from the p21-activated kinase family); PALA, PALB, PALC, PALF, and PALH (various proteins from the PAL/RIM pathway); PACC (terminal transcription factor of the PACC pathway); and CSN1-8 (COP9 signalosome proteins 1 to 8).

The importance of the STRIPAK complex and its subunits in sexual development has been experimentally shown in *S. macrospora*. Deletion of the genes encoding STRIPAK proteins commonly results in similar phenotypes, particularly during the development of the immature ascomata. In Δ*pro11* (167), Δ*pro22* (161), Δ*PP2Ac1* (168), Δ*kin24* (169), and Δ*pro45* (170) mutants, protoascomata are produced normally but do not become pigmented over time and do not develop into mature sexual structures. In strains where *mob3* (171) or *pro11* (161) are deleted, an even more extreme phenotype is apparent, with the complete absence of any sexual tissues. Furthermore, the deletion of *PP2Ac2*, the catalytic subunit of the phosphatase, is lethal (168).

The precise manner in which the STRIPAK complex regulates ascomatal development is not fully understood. However, CLA4, a STRIPAK target might provide some insight into this pathway (166). This protein is a member of the p21-activated kinase family and is important for sexual reproduction in *F. graminearum*, *N. crassa*, and *M. grisea* (172–174). In *S. macrospora* specifically, Δ*cla4* strains produce deformed ascomata and significantly fewer asci and ascospores than wild-type strains (166). The role that CLA4 plays in sexual development in *S. macrospora* is heavily dependent on a phosphorylation site within its catalytic subunit, a site that is directly regulated by STRIPAK. In fact, when this site was mutated so that it could not be dephosphorylated by STRIPAK, the production of asci and ascospores was greatly reduced, producing a phenotype comparable to the Δ*cla4* mutant (166). Further investigation into the other STRIPAK target proteins is likely to identify additional developmental pathways regulated by this complex.

PACC Pathway

A second pathway that appears to be important in the development of the protoascomata in filamentous fungi, particularly in *N. crassa*, is the PACC pathway. This name reflects the final step of the pathway that involves the activation of the PACC transcription factor (175). This pathway has been implicated in pH response, the regulation of xylanase, cellulolytic and endoglucanase activities, and metabolism of glycogen and trehalose in *N. crassa* (176).

The PACC pathway has been particularly well-characterized in *S. cerevisiae* and *A. nidulans*, where it is activated when there are significant pH changes in the environment (Fig. 5) (177–179). The PALH protein acts as the pH sensor and is physically bound to the PALF protein, which is phosphorylated upon sensor stimulation. This results in PALH being released and joining a complex of proteins, including PALA, PALB, PALC, and PACC. This in turn stimulates the activation of PACC via PALB-mediated cleavage. The activated PACC transcription factor is then translocated into the nucleus, where it brings about transcriptional change (175).

The genes that encode the PACC pathway proteins in *A. nidulans* do not appear to play an important role in sexual development. In contrast, genes encoding the six proteins of this pathway have been identified in the *N. crassa* genome, and all are essential for the production of protoascomata (175). Deletion of any of the six genes that encode PACC pathway proteins result in mutants which, when grown on synthetic nitrogen-limited, crossing media, are unable to produce ascogonia or protoascomata. This phenotype was rescued when mutants were grown on medium containing the carbohydrates typically required for sexual development in *N. crassa* (175). Given that the pH-dependent PACC pathway is known to regulate the accumulation and metabolism of glycogen and trehalose in *N. crassa* (180), it is possible that the production of the highly complex protoascomata, which is an energetically intensive process, relies on the metabolism of these energy-rich carbohydrates, hence the requirement of a functional PACC pathway.

The PACC pathway is also essential in *N. crassa* for steps downstream of protoascomatal production (175). In single gene mutants where protoascomatal formation had taken place, fertilization resulted in melanization and growth of the protoascomata into mature ascomata. However, the maturing ascomata did not reach full size and did not harbor mature asci, and no ascospores were produced, a phenotype that could not be rescued under different environmental conditions. This might be explained by the fact that the *S. cerevisiae* Rim1p, a *pacC* homolog, is important for meiosis (181, 182). Given that most of this pathway is conserved in *N. crassa* and *S. cerevisiae* (175), it is likely that the PACC pathway is also important for meiosis in *N. crassa*. Overall, this indicates that the PACC pathway is important in both the initial and the later stages of sexual development.

COP9 Signalosome

The COP9 signalosome (CSN) is another evolutionarily conserved complex that regulates sexual development in fungi. The complex regulates this pathway via its roles in response to oxidative stress, the regulation of redox status and cell wall rearrangement (183). The complex is made up of a variety of proteins, such as those involved in CSN assembly (e.g., PCI-domain proteins) (184, 185) and those with enzymatic activities (e.g., metalloproteases) (184, 186). Together, these proteins act in posttranslational processes, such as protein ubiquitylation and subsequent degradation (186). Via these important enzymatic activities, it is thought that the CSN complex plays an important role as a regulator of multicellularity (184, 187). While the specific manner in which this complex regulates cellular differentiation remains to be understood, it appears that ubiquitin-dependent protein degradation is important for the development of complex, multicellular structures with specific spatial requirements (184).

While the CSN can be made up of as few as six or seven proteins, as is the case in *Schizosaccharomyces pombe* and *N. crassa* (186), the most common conformation of this eukaryotic complex is exemplified by *A. nidulans* (Fig. 6) (184). The *A. nidulans* CSN

is comprised of eight proteins, which are encoded by genes *cnsA* to *csnH*. Four of the eight genes have been functionally characterized with respect to their role in sexual development, including *csnA* (184), *csnB* (184), *csnD* (185), and *csnE* (185). Given the importance of this complex in the development of multicellular structures, it is perhaps not surprising that all four of these genes are important for the production of protoascomata. Furthermore, their deletion results in mutant isolates capable of forming only the initial structures (primordia) associated with sexual development, but these initials do not mature into protoascomata or any other mature sexual structures. Remarkably, even a single gene deletion results in this extreme phenotype, strongly suggesting that the functioning of this complex relies on all of the eight proteins (184, 185).

ASCOSPORE PRODUCTION AND GERMINATION

The final stages of sexual reproduction include the production of the ascospores, their release into the environment, and subsequent germination.

Ascosporegenesis

The production of ascospores relies on multiple rounds of cell division. Meiotic divisions within the ascus result in four haploid nuclei that are typically arranged in a linear conformation, each of which undergo a round of mitosis to produce the final eight ascospores (115). Further ascospore development takes place to ensure the eight spores are arranged, partitioned, and delineated correctly in the ascus.

There are various genes in *A. nidulans* and *N. crassa* that, when deleted, produce empty asci. This phenotype indicates that the appropriate sexual tissues are present but that meiosis cannot be initiated within the mature asci. Four of these genes are *tubB* (188) and *grrA* (189) in *A. nidulans* and *sad1* (190) and *sad2* (191) in *N. crassa*. The two *A. nidulans* genes have very different functions; the *tubB* gene is involved in microtubule assembly (188), while *grrA* plays an important role in protein degradation (189). In contrast, both of the *N. crassa* genes are important for meiotic silencing (190, 191) and, importantly, SAD2 ensures the proper localization and thus functioning of SAD1 (191). Despite difference in their precise functions, each of these genes enables the commencement of the meiotic cycle within the sexual structures.

The *ski8* gene in *S. macrospora* is essential for many different stages of ascosporegenesis, with functions as early as ascomatal production and ascus emergence and as late as meiosis (192). Importantly, it is thought that the SKI8 protein is particularly important for the production of double stand breaks in the DNA during meiosis, since Δ*ski8* mutants do not produce chiasmata, and they undergo meiosis aberrantly with delayed karyogamy. These mutants typically contain many asci in which unfused nuclei are common and mature ascospores have not been produced (192).

The *S. macrospora* STE12, a transcription factor that interacts directly with the MAT1-1-1 protein, is important for ascus and ascospore formation (193). When the *ste12* gene is deleted, mutants produce ascomata that appear normal but with very few asci, and those that are produced have very thin and fragile cell walls that burst easily. Similarly, the number of reproductive structures, including hooked cells and meiosis-specific cell stages, are significantly reduced in mutants compared to wild-type isolates (193).

Several genes that encode proteins involved in autophagy in *S. macrospora* are important for production of asci and ascospores (194–196). Two such examples are *nbr1* and *lon2*, which encode an autophagy cargo receptor and a Lon protease, respectively (194, 195). Given that autophagy results in the reconstitution of cellular components and may thereby act as a mechanism of nutrient provision, particularly during periods of cellular development, it is not surprising that this pathway directly affects the production of highly specialized sexual tissues. The deletion of *nbr1* resulted in the production of immature, malformed asci which have significantly fewer ascospores than wild-type asci (195). Similarly, Δ*lon2* mutants produced fewer ascomata, most of which contained asci housing only immature ascospores (194). Both mutants were more sensitive to stressors as well, with the Δ*nbr1* mutants not producing fruiting bodies under

temperature and nutritional stress and Δ*lon2* mutants not producing fruiting bodies during starvation (194, 195). These results strongly suggest that the autophagous pathway is important in the environmental stress response, while maintaining the nutritional status necessary for sexual development.

In *N. crassa*, the *asd1* gene partially regulates ascosporegenesis once meiosis and mitosis have already taken place (197). This gene encodes a rhamnogalacturonase, which is thought to play a role in the regulation of sexual development, via the production of various polysaccharides. In Δ*asd1* mutants, the asci form normally but despite going through meiosis as usual, the spores do not delineate, and the haploid nuclei remain unpackaged (197). The *sec22* gene plays a similar role in *S. macrospora* where it is involved in ascospore maturation (198). In wild type isolates, the eight spores present in the asci develop at a uniform rate before being ejected together. In Δ*sec22* mutants, however, these spores mature at different rates and some are ejected well before maturation (198).

Germination

Ascospore germination has been studied in detail in *S. macrospora*, and a number of genes that directly influence germination rate have been described. In addition to the role played by the autophagous pathway in the production of asci as described above, autophagy is also important for ascospore germination in *S. macrospora* (196). Two genes, *atg4* and *atg8*, encode autophagy-related proteins, and both directly affect the germination rate of ascospores in this species (196). Deletion of either *atg4* or *atg8* results in a germination rate reduction of about 50%. Since germination involves the growth of a mycelium from a spore, it is reasonable to speculate that autophagy may play an important role in providing nutrients to the germ tube. Given this role, it is not surprising that these two genes are also involved in the development of the maturing ascomata as well (196).

A third gene involved in ascospore germination in *S. macrospora* is *sec22* (198). Interestingly, this gene, which encodes a membrane-associated protein involved in membrane fusion, may also be involved in autophagy (199), further supporting the role of this process in germination. In Δ*sec22* mutants, a mixture of immature and mature spores is released from the asci (198). The immature spores never germinate, and the mature spores germinate at a lower rate than the wild type (198).

As with *atg4* and *atg8*, *sec22* appears to have multiple roles in sexual development, ranging from spore production, pigmentation, and maturation to ascospore germination postrelease (196, 198). Interestingly, deletion of *sec22* resulted in the deregulation of genes involved in numerous developmental pathways, including those putatively involved in vesicle transport (198). Given the large number of diverse molecular processes that rely on this cellular transport pathway, it is not surprising that the *sec22* gene is involved at numerous stages in the sexual cycle.

Another two genes in *S. macrospora* that influence germination are important for G-protein-coupled signal transduction (152). These genes, *gsa3* and *sac1*, encode the Gα-subunit and the adenylyl cyclase protein, respectively. Upon activation of the GPCR, the Gα-subunit activates the adenylyl cyclase, thereby initiating an appropriate transduction cascade, which, in this case, would result in the formation of the germ tube. Deletion of the *gsa3* gene results in normal ascomata with ascospore-bearing asci, but only approximately 5% of these ascospores germinate (152). Similarly, Δ*sac1* mutants also exhibit a significant decrease in germination but also produce much smaller ascomata during sexual development (152).

LOOKING TO THE FUTURE

Sexual reproduction in fungi has been studied for decades and the progress that has been made is nothing short of remarkable. From the original descriptions of different fungal fruiting bodies and mating behaviors to the identification of the diffusible mating pheromones and the elucidation of the *MAT* genes, our understanding of this

pathway has grown considerably, stretching from physiological observations to the characterization of the underlying genetic mechanisms that govern the process.

Studies on the pathways that govern sexual reproduction relied heavily on the functional characterization of single genes and their direct effect on the sexual capabilities of model fungi. This approach has proven very useful to shape our understanding of this process but is plagued by some significant drawbacks. The first of these is that such a complex pathway is necessarily governed by extensive, multifaceted gene and protein networks that need to be characterized with respect to, and within the context of, one another. In addition, a significant level of redundancy often exists when it comes to the function of these genes, with two or three proteins having overlapping functions and thus compensating for one another. This essentially masks the effects and importance of certain genes when they are knocked out. Lastly, it is often not possible to fully characterize the function of a gene if it is essential for numerous steps in a particular pathway. For example, if a gene is essential for both fertilization and the production of asci, its role in ascus production will be masked because the block in fertilization will preclude any further sexual development. Thus, single gene knockouts do not always accurately allow for the characterization of the functions of these genes and cannot be used to describe all forms of interconnected complexity. The advent of more powerful tools such as CRISPR-Cas9 and NGS promises to change this. Apart from the ability to produce single gene knockouts, CRISPR can efficiently produce multigene knockouts in a single organism, allowing complex pathways to be better dissected. With transformation protocols becoming more prevalent across the Pezizomycotina, functional studies in underrepresented taxa will become an exciting source of new information in future.

Currently, extensive data are available linking the functions of individual genes and genetic pathways to various steps of the sexual cycle in model species such as *N. crassa*, *A. nidulans*, *S. macrospora*, and the other species discussed above (Fig. 3). However, a significant step forward in our understanding of sexual development in the Pezizomycotina has been the broadening of the field to include non-model species. Over the past few years, the sexual cycles of non-model species like *V. virens* (60, 65) and *H. omanensis* (64) have also been subjected to genetic inquiry. This has enabled the comparison of gene function of homologs from different species, which in turn enables further dissection of the evolutionary conservation of these processes. This is particularly important if we seek to continue using fungi as multicellular representatives of the Eukarya and extrapolating the conclusions drawn from fungi to other eukaryotes.

A number of gaps exist in the literature (Fig. 3), paving the way for future investigations and a better understanding of sexual development in the Pezizomycotina. Some key genes and pathways remain to be elucidated, including the identification of the **a**-factor-like pheromone in the Eurotiomycetes and related fungi, as well as the targets of the evolutionarily conserved protein complexes, STRIPAK and CSN. In addition, much work remains to done on how various environmental cues regulate sexual development. For example, the effect of light on the sexual cycles of *N. crassa* and *A. nidulans* is very well understood both in terms of the environmental stimuli and the genetic and physiological responses. However, the importance of other environmental factors such as pH, temperature, and nutrient availability are less well understood in filamentous species, particularly with regard to the genes and proteins involved in regulating these pathways.

The role of posttranscriptional and posttranslational modification clearly requires further investigation. It is known, for example, that RNA editing is important for ascosporegenesis and ascus maturation in both *F. graminearum* (200) and *N. crassa* (201). Similarly, posttranslational modifications are known to be essential for the functioning of both pheromone factors. However, the extent to which these types of modifications are necessary and essential for the sexual cycle is not yet known. Whether such of modifications occur in all of the Pezizomycotina also remains a topic open for debate.

CONCLUDING REMARKS

Fungi provide fascinating and effective models for studies focused on elucidating a process as complicated as sexual reproduction. Fungi exhibit numerous distinct and, in some cases, unusual sexual strategies and there are a growing number of model species available for study and comparison. The fungi also include an unusually wide diversity of morphologies, sexual structures, and preferred environments, providing a wide array of opportunities to study sexual development in depth.

New and emerging technologies are driving opportunities to better understand sexual reproduction in the fungi. For example, genome editing, whole-genome sequencing, and comparative transcriptomics are becoming increasingly accessible and available for such investigations. These are likely to reveal a substantially improved understanding of sexual development in a diverse array of model and non-model fungal species. The results will make it possible to extrapolate emerging knowledge beyond the fungal kingdom to the other eukaryotes.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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Michael J. Wingfield, the founding director of the Forestry and Agricultural Biotechnology Institute, serves as an advisor to the University of Pretoria Executive. His research focuses on the health of forest trees, having published widely on this topic together with more than a hundred past Ph.D. students. He served a five-year term as President of the International Union of Forestry Research Organizations (IUFRO) and has been widely recognized for his research, including receiving the Kwame Nkrumah Science Award from the African Union and being awarded honorary doctorates from the University of British Columbia (Canada) and North Carolina State University (USA).

Brenda D. Wingfield has made the study of the global movement and evolution of fungal pathogens, particularly those on trees, her research focus for the past 30 years. Professor Wingfield holds the DSI-NRF SARChI chair in Fungal Genomics. She pioneered fungal genomics at the University of Pretoria and was responsible for the first fungal genome to be sequenced in Africa. Her success is reflected in the internationally recognized work of many of her past Ph.D. students. She is currently the Secretary General of the International Society of Plant Pathology and was the vice president of the Academy of Science of South Africa.