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Functional Characterization of *MAT1-1*-Specific Mating-Type Genes in the Homothallic Ascomycete *Sordaria macrospora* Provides New Insights into Essential and Nonessential Sexual Regulators▽†

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Mating-type genes in fungi encode regulators of mating and sexual development. Heterothallic ascomycete species require different sets of mating-type genes to control nonself-recognition and mating of compatible partners of different mating types. Homothallic (self-fertile) species also carry mating-type genes in their genome that are essential for sexual development. To analyze the molecular basis of homothallism and the role of mating-type genes during fruiting-body development, we deleted each of the three genes, *SmtA-1* (*MAT1-1-1*), *SmtA-2* (*MAT1-1-2*), and *SmtA-3* (*MAT1-1-3*), contained in the *MAT1-1* part of the mating-type locus of the homothallic ascomycete species *Sordaria macrospora*. Phenotypic analysis of deletion mutants revealed that the PPF domain protein-encoding gene *SmtA-2* is essential for sexual reproduction, whereas the α domain protein-encoding genes *SmtA-1* and *SmtA-3* play no role in fruiting-body development. By means of cross-species microarray analysis using *Neurospora crassa* oligonucleotide microarrays hybridized with *S. macrospora* targets and quantitative real-time PCR, we identified genes expressed under the control of *SmtA-1* and *SmtA-2*. Both genes are involved in the regulation of gene expression, including that of pheromone genes.

Sex, one mechanism of the genetic diversity of species, is ubiquitous across kingdoms. To avoid self-crossing, genetic barriers have evolved that prevent selfing, and these often culminate in sexual dimorphism. In filamentous ascomycetes, sexual dimorphism is almost nonexistent, and in many cases individuals are hermaphrodites. Here, sex is determined genetically by a sex-specific region in the genome known as the mating-type locus (*MAT*) (10).

Fungi exhibit two different sexual life styles, homothallism (self-fertility) and heterothallism (self-sterility) (46). This phenomenon was first discovered by Blakeslee in the group of zygomycetes, a lineage that diverged early within the fungal kingdom (5). Only recently was the sequence of the mating-type locus of *Phycomyces blakesleeanus* (*Zygomycota*) discovered (36). It was shown that each mating-type locus contains one single gene coding for a protein with a high-mobility group (HMG) (31) (Fig. 1). Both genes show low-level amino acid similarity and confer the ability to mate as either a *MAT* (−) or a *MAT* (+) strain.

Similarly, heterothallic ascomycetes contain a single mating-type locus with two alternate alleles. The DNA sequences at the mating-type locus in individuals of different mating types show almost no homology. To emphasize the dissimilarity between and the different origins of the genes of different mating-type loci, they have been termed idiomorphs instead of alleles (49).

In ascomycetes, mating is best characterized at the molecular level in the budding yeast *Saccharomyces cerevisiae*. The *MAT* idiomorphs, *MAT***a** and *MAT*α, encode regulatory proteins which, in combination with other transcription factors, are responsible for a distinct pattern of expression in the three yeast cell types: haploid *MAT***a** and *MAT*α cells and diploid *MAT*α/**a** cells (33). Haploid yeast cells sense the presence of a potential mating partner by recognizing pheromones specifically secreted by cells of the opposite mating type (4).

The *MAT*α mating-type locus consists of two genes, α*1* and α*2* (1). The α1p protein carries a characteristic DNA-binding domain called the α domain and is a positive regulator of transcription of α-specific genes. The product of the α*2* gene is a homeodomain protein that acts as a negative regulator of transcription of **a**-specific genes (2). The *MAT***a** locus consists of two genes, but only ***a1*** encodes a functional protein with a homeodomain. The **a**1 protein plays no role in mating regulation in haploid **a** cells, since **a**-specific genes are constitutively expressed in the absence of α2p. However, **a**1p forms a heterodimer with α2p in diploid cells. The **a**1/α2 heterodimer represses the expression of haploid-specific genes. The gene ***a2*** encodes a nonfunctional version of α2 (18, 33). However, the coding capacity of *S. cerevisiae MAT* loci is not representative of other ascomycetes (16, 35). The *MAT* locus of *S. cerevisiae* lacks a gene coding for an HMG domain protein, which is present in the *MAT* loci of ascomycetes ranging from filamentous ascomycetes (*Pezizomycotina*) to the fission yeast *Schizosaccharomyces pombe* (*Taphrinomycotina*) (76) (Fig. 1).

In heterothallic *Pezizomycotina*, the single mating-type locus conferring mating behavior also consists of dissimilar DNA sequences (idiomorphs). Instead of *MAT*α and *MAT***a**, the

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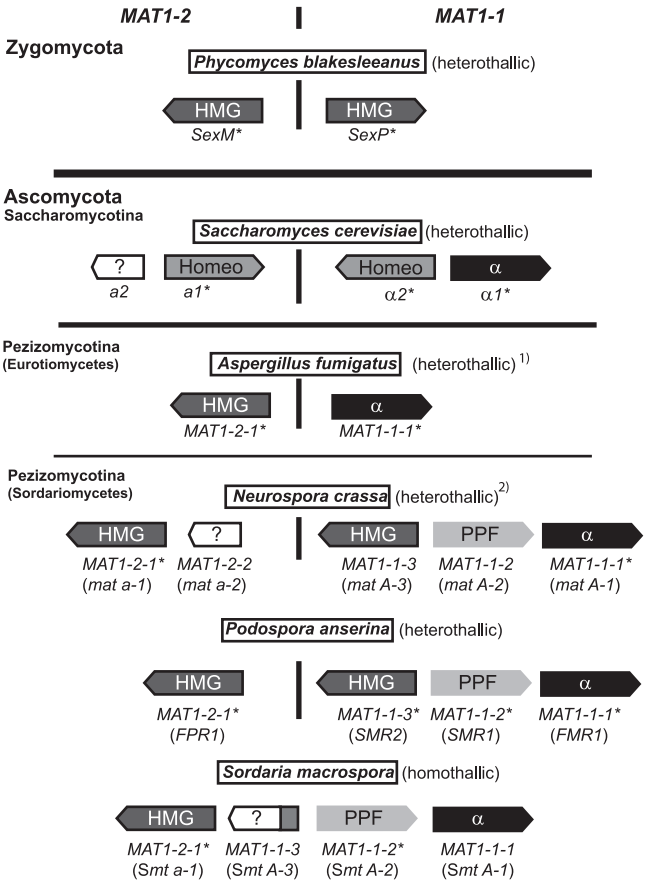


FIG. 1. Schematic comparison of mating-type loci from heterothallic and homothallic fungi. The arrowed boxes represent the orientation and size of the ORFs in the mating-type locus. Species-specific names and standard nomenclature are given. Known functional domains of the encoded mating-type proteins are given, and genes containing the same functional domain are the same color. α, α domain; HMG, HMG domain; Homeo, homeodomain; PPF, PPF domain; ?, no known domain. Genes essential for sexual development are indicated with an asterisk. Superscripts: 1, homothallic *A. nidulans* carries two unlinked counterparts of *MAT1-1-1* and *MAT1-2-1*; 2, the homothallic *Neurospora* species *N. africana*, *N. dodgei*, *N. galapagosensis*, and *N. lineolata* contain only *MAT1-1*-specific genes.

mating types of *Pezizomycotina* were originally described as *MAT*− and *MAT*+ or *mat A* and *mat a* and later renamed by Turgeon and Yoder (78). *MAT1-1* corresponds to *MAT*α of *S. cerevisiae*, and *MAT1-2* corresponds to the *MAT***a** idiomorph of *S. cerevisiae*. Without exception, the *MAT1-1* idiomorph of *Pezizomycotina* contains a gene encoding a protein with an α domain, whereas *MAT1-2* carries a gene encoding a protein with an HMG domain. Heterothallic members of the class *Eurotiomycetes*, such as *Aspergillus fumigatus*, have this simple mating-type structure (Fig. 1). In addition to these two genes, other genes may be present at the *MAT* locus (16) (Fig. 1). With regard to the function of *MAT* genes, two heterothallic members of the class *Sordariomycetes*, *Neurospora crassa* and *Podospora anserina*, are the best-characterized species within the subphylum *Pezizomycotina*. Their mating-type loci are similar in structure (Fig. 1).

The *MAT1-2* (*mat a*) locus of *N. crassa* comprises two genes, *MAT1-2-1* (*mat a-1*) and *MAT1-2-2* (*mat a-2*) (61, 74). While the function of *MAT1-2-2* is unknown, the HMG domain gene *MAT1-2-1* encodes the main regulator of sexual reproduction in *MAT1-2* strains (11).

The *MAT1-1* (*mat A*) idiomorph of *N. crassa* contains three genes, the α domain gene *MAT1-1-1* (*mat A-1*), *MAT1-1-2* (*mat A-2*), and the HMG domain gene *MAT1-1-3* (*mat A-3*) (25, 29). The MAT1-1-1 polypeptide is the major regulator of *MAT1-1* mating functions (6, 25, 29, 71). The gene *MAT1-1-2* encodes a protein without a known DNA-binding motif. However, it contains a conserved region with three invariant residues, histidine, proline, and glycine, which was initially called the HPG domain (17). MAT1-1-2 homologues of members of the genus *Diaporthe*, however, lack the conserved His, Pro, and Gly residues but, like all other MAT1-1-2 proteins, have two invariant prolines and one phenylalanine residue. Therefore, the name of the region has recently been changed to the PPF domain (16, 39). PPF domain proteins are present in the *MAT1-1* loci of all known *Sordariomycetes*, but no homolog has been found outside this taxon (16, 77) (Fig. 1).

MAT1-1-2 and *MAT1-1-3* deletion mutants of *N. crassa* show only slightly decreased fertility and no distinguishable vegetative phenotype. Deletion of both genes results in strongly decreased fertility, but the mutants are still able to produce viable ascospores (24). Other than in *N. crassa*, deletion of *MAT1-1-2* in *P. anserina* leads to a complete arrest of fruiting-body development (16, 77). MAT1-1-2 of *P. anserina* (PaMAT1-1-2) shows about 20% identity to its counterpart in *N. crassa* and was proposed to be a DNA-binding protein, but later investigations indicated a cytosolic localization (15, 17, 25).

The filamentous ascomycete *Sordaria macrospora*, a close relative of *N. crassa*, is homothallic and therefore does not need a mating partner to complete the sexual cycle. Nonetheless, the genome of *S. macrospora* contains a mating-type locus (66). The locus is similar to both the *MAT1-1* (*mat A*) and *MAT1-2* (*mat a*) idiomorphs of *N. crassa*. It harbors four mating-type genes, the HMG domain gene *Smta-1* (*MAT1-2-1*), the small gene *SmtA-3* (*MAT1-1-3*), the PPF domain gene *SmtA-2* (*MAT1-1-2*), and the α domain gene *SmtA-1* (*MAT1-1-1*), and displays a high degree of sequence similarity to the corresponding mating-type genes of *N. crassa* and to mating-type genes of other *Sordariaceae* (61, 66) (Fig. 1). Interestingly, SMTA-3 has a chimeric character and contains sequence similar to the *N. crassa* MAT1-2-2 and MAT1-1-3 proteins but lacks the characteristic HMG domain of MAT1-1-3. Thus, *SmtA-3* encodes a protein with no known functional domain. Mating-type genes of *S. macrospora* have been demonstrated to be functional in heterothallic *P. anserina* (59, 66), and the HMG domain gene *Smta-1* has been shown to be essential for sexual development in *S. macrospora* (65).

As in *S. cerevisiae*, mating-type-encoded transcription factors of heterothallic filamentous ascomycetes are supposed to act directly or indirectly as transcriptional regulators on the mating-type-specific expression of pheromone and pheromone receptor genes (2, 42). In fact, it has been demonstrated for heterothallic filamentous ascomycetes that pheromone signaling enables cells of opposite mating types to detect each other (14, 40). Interestingly, two different pheromone precursor genes and two pheromone receptor genes have been found in homothallic *S. macrospora* and have been shown to be involved

in the sexual development of *S. macrospora* (48, 60, 63). Moreover, a cross-species microarray analysis using *N. crassa* microarrays identified many genes up- or downregulated in a ΔSmta-1 deletion mutant, including the a-factor-like *ppg2* gene, which is 500-fold downregulated in ΔSmta-1 compared to the wild type (WT) (65).

The functions of the other three *MAT1-1* mating-type genes in *S. macrospora* are unknown. In this study, we deleted the *MAT1-1*-specific genes *SmtA-1*, *SmtA-2*, and *SmtA-3* and analyzed the phenotype of the mutants. While *SmtA-2* is essential for fruiting-body and ascospore development, *SmtA-1* and *SmtA-3* appear to play no role in vegetative growth or sexual reproduction. Thus, similar to the situation in the phycomycete *P. blakesleeanus* but contrary to all other studied ascomycetes, sexual reproduction in *S. macrospora* is not dependent on an α domain protein. Only one HMG domain protein and one PPF domain protein are necessary for full fertility in this ascomycete. Additionally, cross-species microarray hybridizations were used to identify genes that are differentially regulated in the ΔSmtA-1 and ΔSmtA-2 mutants, among them the pheromone precursor genes.

MATERIALS AND METHODS

Strains and culture conditions. For cloning and propagation of recombinant plasmids, *Escherichia coli* strain SURE was used under standard culture conditions (70; Stratagene, La Jolla, CA). *S. cerevisiae* strain PJ69-4A (38) was grown in YEPD full medium or SD minimal medium lacking uracil (72). *S. macrospora* strains were cultivated on cornmeal medium (BMM) or complete medium (CM) (21, 23). For RNA extraction, strains were grown in floating cultures on fructification medium (SWG) at 24°C as described by Nowrousian et al. (55). The growth rate and dry weight of *S. macrospora* WT strain S48977, as well as of the different deletion mutants, were measured according to Nolting and Pöggeler (50).

Generation of mating-type gene deletions in *S. macrospora*. Deletion constructs of the different mating-type genes were created utilizing homologous recombination in yeast (13). The 5′ and 3′ regions of the respective mating-type gene were amplified by using corresponding primers 5f/5r and 3f/3r, respectively (see Table S1 in the supplemental material). Within these PCRs, specific 29-bp overhangs were added to the 5′ and 3′ flanks, respectively. These overhangs were homologous to yeast plasmid pRS426 (12) and the hygromycin resistance cassette (*hph*), respectively. The *hph* cassette was amplified from plasmid pCB1003 (9) with primers hph-f and hph-r (see Table S1 in the supplemental material). All three different PCR fragments and the linearized (EcoRI/XhoI) vector pRS426 were transformed into *S. cerevisiae* strain PJ69-4A, where homologous recombination took place. The resulting deletion plasmids, pRS_ΔSmtA1, pRS_ΔSmtA2, pRS_ΔSmtA3, and pRS_ΔSmtA2/3 (see Table S2 in the supplemental material), were isolated according to the protocol of Colot et al. (13) and afterwards used as a template to amplify the deletion cassette with primer pairs Ba1-5f/Ba1-3r, Ba2-5f/Ba2-3r, Ba3-5f/Ba3-3r, and Ba2-5f/Ba3-3r, respectively. The PCR fragments obtained were transformed into the *S. macrospora* Δku70 strain (62) to facilitate deletion by homologous recombination. In double-deletion strain ΔSmtA2/3, both genes were replaced by homologous recombination with the *hph* cassette. The linear fragment was obtained by using the 5′ flank of *SmtA-3* and the 3′ flank of *SmtA-2*. Fungal protoplasts were transformed either with linear PCR fragments generated from deletion plasmids or with complementation plasmids (see Table S2 in the supplemental material). Transformation of *S. macrospora* was performed as described by Nowrousian et al. (53). Plasmids containing no resistance marker for *S. macrospora* were cotransformed with pRSnat, a derivative of plasmid pRS426. The nourseothricin resistance gene *nat1*, under the control of the *Aspergillus nidulans trpC* promoter, was amplified from plasmid pD-NAT1 (43) and inserted into MunI-linearized plasmid pRS426, resulting in pRSnat. Transformants were selected on either hygromycin B (110 U/ml)- or nourseothricin (50 μg/ml)-containing CM.

As fungal transformants are often heterokaryotic and carry both transformed and nontransformed nuclei, single spore isolates were generated by crossings with the *S. macrospora* spore color mutant r2 (S67813) or fus1-1 (S23442) from the strain culture collection of the Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr University Bochum, Bochum, Germany. To complement the phenotype of ΔSmtA-2 and ΔSmtA-2/3, plasmids pRS_Ba2 and pRS_Ba3 (see Table S2 in the supplemental material) were constructed by amplifying the 5′ and 3′ regions and the entire coding regions of *SmtA-2* and *SmtA-3* with primer pairs Ba2-5f/Ba2-3r and Ba3-5f/Ba3-3r from genomic WT DNA, respectively. Amplicons were integrated into the vector pRSnat by homologous recombination in *S. cerevisiae*.

TABLE 1. Segregation of markers in the progeny of different-mating-type mutants and the self-fertile spore color mutant fus1-1, which produces brown spores

Mutant strain	No. of progeny of each phenotype[a]				Total
	Parental		Recombinant		
	HygR/fus$^+$	HygS/fus$^-$	HygS/fus$^+$	HygR/fus$^-$	
ΔSmta-1	24	31	28	22	105
ΔSmtA-1	29	17	32	21	99
ΔSmtA-2	16	21	23	15	75
ΔSmtA-3	31	25	23	24	115

[a] Markers that were tested for are hygromycin B resistance (HygR/HygS for resistance/sensitivity) and spore color (fus$^+$/fus$^-$ for black/brown spores). The segregation pattern fits a 1:1:1:1 ratio; *P* value, >0.015.

Preparation of nucleic acids, hybridization protocols, and PCR. Isolation of genomic DNA from *S. macrospora* was done as previously described (66). Verification of homologous recombination at the locus of the target gene was done by amplification of the 5′ region with primers Ba1Ko-f, Ba2Ko-f, and Ba3Ko-f/trpC1 and of the 3′ region with hph-3/Ba1Ko-r, Ba2Ko-r, and Ba3Ko-r (see Table S1 and Fig. S1 in the supplemental material), respectively, with HotStarTaq DNA polymerase (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Southern blotting was done according to standard techniques (70). Hybridization was done with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Mannheim, Germany). DNA probes were obtained by PCR with primers hph-f and hph-r (see Table S1 in the supplemental material), and labeling and detection were done according to the manufacturer's protocol.

Crossing experiments. Mycelial plugs (2 mm) of strains carrying either a mating-type gene mutation or a mutation leading to a change in spore color (strain fus1-1) were placed on opposite sides of a plate containing full medium (BMM) (Table 1). After 7 days of incubation at 27°C, perithecia were isolated from the touching zone of both mycelia. From perithecia containing ascospores of both spore colors (WT/fus1-1), 100 spores were isolated and, after germination, transferred to medium containing hygromycin as a selection marker. The different progeny (resistant/nonresistant, WT/fus) were counted and analyzed according to Lee et al. (45). Crossing experiments between different mating-type mutants were done using one strain carrying only the mating-type gene mutation and another strain carrying a spore color mutation (fus1-1).

Quantitative real-time PCR. After 5 days of growth on SWG, RNA was prepared using Trizol (Invitrogen Life Technologies) according to Elleuche and Pöggeler (20) and the integrity of the RNA was verified by agarose gel electrophoresis. Reverse transcription of 1 μg total RNA for real-time PCR was done according to Nowrousian et al. (55), using 400 U Superscript III (Invitrogen) and 0.25 M deoxynucleoside triphosphates. Real-time PCR was performed in an Eppendorf Realplex2 Mastercycler with qPCR Master-Mix Plus for Sybr green (Eurogentec, Belgium) in a volume of 20 μl. PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis. For the specific primer pairs used for real-time PCR, see Table S1 in the supplemental material. Amplification of a part of the SSUr RNA with primer pair SSU-f/SSU-r was used as a reference for the normalization of threshold cycle values (65). For each strain and primer pair, real-time experiments were carried out thrice in triplicate with biologically independent samples.

Microarray hybridization. *N. crassa* microarrays with 10,910 70-mer oligonucleotides corresponding to predicted open reading frames (ORFs) were used (75). RNA isolation was accomplished as described previously (55), after incubation of *S. macrospora* strains for 4 and 5 days on SWG. Poly(A) RNA was isolated with the polyATtract kit (Promega) according to the manufacturer's protocol. Microarray targets from *S. macrospora* were made by reverse transcrip-

tion with Superscript II reverse transcriptase (Gibco) in the presence of aminoallyl-dUTP (Sigma) from 1-μg aliquots of poly(A) RNA.

The dyes Alexa555 and Alexa647 (Invitrogen catalog no. A-32755) were coupled to the cDNA in the presence of 7.5 mg/ml sodium bicarbonate buffer. The cDNA was subsequently cleaned by using the Illustra Cyscribe GFX purification kit (Amersham/GE catalog no. 27-9606-02).

Before prehybridization slides were treated with 600 mJ UV light and pretreated with the Pronto Background Reduction kit (Corning; catalog no.40029).

Slides were prehybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–1.0% bovine serum albumin for 1 h at 42°C, washed, and spin dried. Following slide prehybridization, labeled cDNA was resuspended in 36 μl of hybridization solution (25% formamide, 5× SSC, 0.1% SDS, 0.1 μg/μl single-stranded DNA, 0.2 μg/μl tRNA) and the suspension was heated at 95°C for 5 min and subsequently transferred into the space between a microarray slide and a LifterSlip cover glass (Erie Scientific, Portsmouth, NH). Hybridization was carried out for 16 h at 42°C in a Boekel InSlide Out Hybridization Oven, and unbound DNA was washed off according to the manufacturer's instructions for Corning Ultra GAPS slides. A GMS 418 Scanner was used to acquire images, and Scanalyze software (Michael Eisen, http://rana.lbl.gov/EisenSoftware.htm) was used to quantify hybridization signals.

The resulting data files were further analyzed with Excel (Microsoft), Bioconductor project (28), and the MultiExperimentViewer (MeV) (69). Statistical analysis was carried out in the R computing environment (version 2.7.2). using the Linear Model for Microarray Data package (73). Data were normalized and scaled between the arrays using variance stabilization and calibration for microarray data (34), and the differential expression of genes was determined by an empirical Bayes approach within LIMMA. The R protocol was used according to Nowrousian et al. (52) with targets (WT, ΔA1, ΔA2) specified in file target.txt.

Genes were defined as being differentially regulated if they showed a $\log_2$ ratio of >1 or ≤ 1 and a P value of <0.05.

Microscopic investigations. Transformants were inoculated on microscope slides coated with a thin layer of solid BMM. After incubation at 24°C, the samples were analyzed using an AxioImager M1 microscope (Zeiss, Jena, Germany). Images were taken with a Photometrics CoolSNAP HQ^2 camera (Roper Scientific, Photometrics, Tucson, AZ). Recorded images were edited with MetaMorph (Visitron Systems GmbH, Puchheim, Germany) and Adobe Photoshop CS2.

Microarray data accession number. Details of experimental procedures, raw data, and results of statistical analysis of microarray hybridizations were submitted to the public repository ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) and can be retrieved under accession no. E-MEXP-2600.

RESULTS

Generation and phenotypic analysis of *S. macrospora* mating-type gene deletion strains. We generated the ΔSmtA-1, ΔSmtA-2, and ΔSmtA-3 mutant strains to determine the role of the *MAT1-1*-specific mating-type genes. Because the *N. crassa* Δ*MAT1-1-2*/*MAT1-1-3* double-deletion mutant displays reduced fertility compared to the single-deletion mutants (24), we constructed a ΔSmtA-2/3 double-deletion mutant of *S. macrospora*. All deletion strains were generated by gene replacement as described in Materials and Methods. Linear fragments were amplified by PCR and transformed into the *S. macrospora* Δku70 strain, which is impaired in the repair of DNA double-strand breaks and has been shown to be an ideal recipient strain for gene targeting experiments (62). Because *S. macrospora* primary transformants are often heterokaryotic, we isolated single spore isolates to segregate the WT and mutant alleles of the *MAT* genes in a Δ*ku70* background. Strains that were homokaryotic for the desired *MAT* gene deletion and carried the *ku70* deletion were further crossed against spore color mutant strain fus1-1. Using conventional genetic analysis, we isolated hygromycin-resistant spores without the Δ*ku70* (nourseothricin-resistant) background, indicating that the *MAT* locus is not closely linked to *ku70* and that none of the *MAT1-1*-specific genes is essential.

PCR amplification with primer pairs specific for external flanking regions in combination with primers specific for the integrated *hph* cassette confirmed the integration of the hygromycin resistance cassette in the desired *MAT* gene and the absence of WT nuclei in the single-spore isolates (see Fig. S1 in the supplemental material). Southern blot hybridization with an immunolabeled probe specific for the *hph* cassette also confirmed the homologous integration without the presence of any heterologous integrated copies of the deletion construct (see Fig. S1 in the supplemental material).

Compared to the WT, the single-deletion strains and the ΔSmtA-2/3 double-deletion strain showed no change in vegetative morphology, growth rate (mean, 1.5 cm/day on BMM at 28°C), or dry weight (mean, 3.5 g after 5 days on BMM at 28°C). The development of sexual reproductive structures in the different mutant strains was analyzed on fructification medium (SWG). All of the mutants showed the formation of ascogonia after 3 days, and these developed into fruiting-body precursors (protoperithecia) after 5 days (Fig. 2). The ΔSmtA-1 and ΔSmtA-3 mutant strains produced the same number of perithecia as the WT, containing mature, viable ascospores at day 7 of development. However, even after incubation for a longer time, mature perithecia were not observed in the ΔSmtA-2 mutant strain or in the ΔSmtA-2/3 double-mutant strain (Fig. 2). The ΔSmtA-2 and ΔSmtA-2/3 mutant strains are blocked in sexual development at the stage of early protoperithecium formation and are therefore sterile. Both mutants produced only protoperithecia with a diameter of <100 μm that did not contain any croziers or ascus initials (data not shown).

The phenotype of the ΔSmtA-2 and ΔSmtA-2/3 strains is similar to that of sterile *S. macrospora* pro mutants blocked at the stage of protoperithecium formation and mutant ΔSmta-1, which have been described previously (47, 52, 55, 64, 65). Complementation analysis of ΔSmtA-2 and ΔSmtA-2/3 was performed by transformation with a plasmid carrying a genomic fragment comprising the *SmtA-2* or *SmtA-3* gene under the control of its own 5′ and 3′ regulatory elements, resulting in strain ΔSmtA-2:*SmtA-2*^{ect}, ΔSmtA-2/3:*SmtA-2*^{ect}, or ΔSmtA-2/3:*SmtA-3*^{ect} carrying *SmtA-2* or *SmtA-3* integrated ectopically under the control of the native promoter. As shown in Fig. 2, the sterile phenotype of ΔSmtA-2 is complemented by the reintroduction of *SmtA-2*. The same is true for ΔSmtA-2/3, while the reintroduction of *SmtA-3* into ΔSmtA-2/3 causes no change in phenotype in independent transformation experiments (data not shown). Therefore, the sterility of ΔSmtA-2/3 is clearly due to the deletion of *SmtA-2*.

Crosses between the mating-type mutants and a self-fertile spore color mutant strain (fus1-1) were set up to determine whether the fertile (ΔSmtA-1 and ΔSmtA-3) and sterile (ΔSmtA-2 and ΔSmta-1) mating-type mutant strains were able to outcross to the tester strain. All outcrosses with the self-fertile tester strain produced mature hybrid perithecia in the contact zone. The number of fertile asci per perithecium (120 to 150) was about the same as in the WT. The perithecia contained asci with complete tetrads of eight ascospores that segregated in each case in a 1:1 ratio for resistance to hygromycin B and for brown spore color (Table 1).

In *S. macrospora*, different sterile mutants are usually able to outcross with each other; after hyphal fusion, the resulting

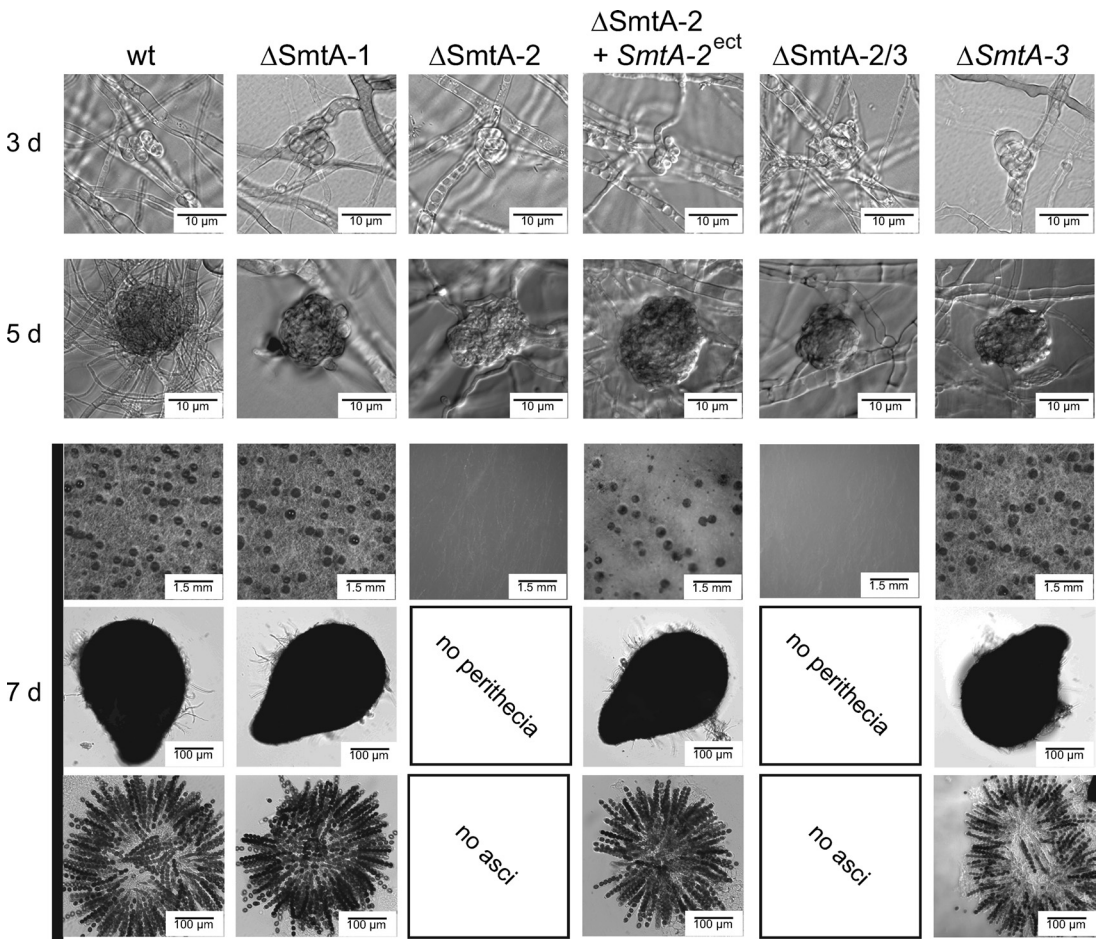


FIG. 2. Sexual development of the WT and deletion mutants ΔSmtA-1, ΔSmtA-2, ΔSmtA-2/3, and ΔSmtA-3. Note the complete absence of perithecia in ΔSmtA-2 and ΔSmtA-2/3 and the WT-like development of ΔSmtA-2 carrying an ectopically integrated *SmtA-2* copy. Strains were grown on SWG at 24°C, and pictures of the sexual structures ascogonia, protoperithecia, and perithecia and asci (not ΔSmtA-2 and ΔSmtA-2/3) were taken after 3, 5, and 7 days of growth on SWG.

heterokaryons contain a functional copy of each mutated gene, which in most cases is sufficient to allow fruiting body development. As expected, crosses between different mating-type mutants showed that ΔSmtA-2 and ΔSmta-1 are able to outcross with the fertile mating-type mutants ΔSmtA-1 and ΔSmtA-3 and produce mature perithecia and viable ascospores. However, crosses between ΔSmtA-2 and ΔSmta-1 resulted in no perithecia or ascospores (data not shown). This means that these two mutations cannot complement each other. One reason for this might be that both proteins are essential for hyphal fusion and therefore the two mutants are not able to form a heterokaryon and thus remain sterile. Another possibility is that the mutants are able to form a heterokaryon but both genes must be present in the same nucleus (in contrast to presence in the same cytoplasm, which would be the case in a heterokaryon) to be functional.

Transcriptional expression of pheromone genes and their cognate receptors. It has been shown that pheromone precursor genes and their cognate receptors are involved in sexual development in *S. macrospora* (48). In the heterothallic relative *N. crassa*, pheromone precursor genes are expressed in a mating-type-specific manner. The homolog of the *S. macrospora* a-factor-like *ppg2* gene, *mfa-1*, is expressed in *MAT1-2* (*mat a*) strains, whereas *ccg-4*, the homolog of *S. macrospora* α-factor-like *ppg1*, is expressed in *MAT1-1* (*mat A*) strains (6). Because ΔSmtA-2 has a sterile phenotype and homologs of α domain protein SMTA-1 are involved in sexual development in other ascomycetes (16), we examined the effects of the α domain protein SMTA-1 and the PPF domain protein SMTA-2 on the expression of the pheromone and receptor genes. We used quantitative real-time PCR to compare the mRNA expression levels of the four genes in ΔSmtA-1 and ΔSmtA-2 versus the WT strain. Real-time PCR experiments were done in triplicate with RNA prepared from mycelia independently grown for 5 days on SWG (Fig. 3). We previously showed that pheromone and receptor genes are well expressed at this time of development (48).

In the ΔSmtA-1 mutant, the transcript levels of *ppg1* and *ppg2* were significantly downregulated compared to those in the WT strain. On average, the amounts of the *ppg1* and *ppg2* mRNAs were reduced about 60-fold and about 16-fold, respectively. In contrast, the transcript levels of *ppg2* in ΔSmtA-2 were increased significantly (20-fold) compared to those in the WT strain, whereas *ppg1* was not regulated differently (Fig. 3).

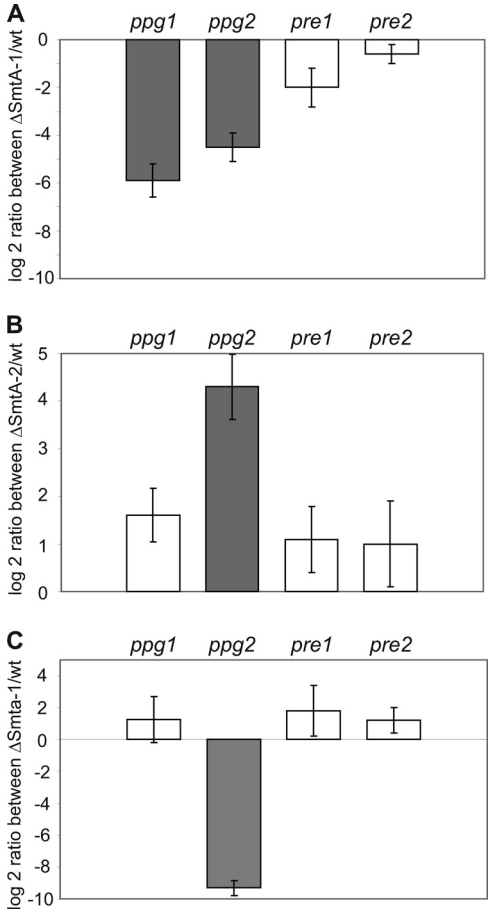


FIG. 3. Quantitative real-time PCR analysis of the pheromone precursor genes *ppg1* and *ppg2* and their cognate receptors *pre1* and *pre2* in ΔSmtA-1 (A), ΔSmtA-2 (B), and ΔSmta-1 (C) compared to the WT. Ratios are given as logarithmic values (base 2) and are means of three independent experiments. Means were calculated with REST (58). The pheromone genes *ppg1* and *ppg2* in ΔSmtA-1 and *ppg2* in ΔSmtA-2 (dark bars) are significantly regulated (P values of 0.001, 0.008, and 0.001, respectively, determined with REST [58]). Shown are averages of three independent experiments ± the standard deviation.

These results indicate that the mating-type protein SMTA-1 acts as a positive regulator of the expression of both pheromone precursor genes *ppg1* and *ppg2*, whereas SMTA-2 has negative effects on the expression of *ppg2*. Similar to SMTa-1 (65), neither of the mating-type proteins had a clear effect on the expression of the pheromone receptors (Fig. 3).

Cross-species microarray analysis of ΔSmtA-1 and ΔSmtA-2. Earlier, we found that cross-species microarray hybridizations with *S. macrospora* targets on *N. crassa* microarrays can be used to identify developmentally regulated genes of *S. macrospora* since the overall identity between the exon sequences of the two species is about 89.5% (55, 56, 65). To identify differentially regulated genes in the mating-type mutants, we used cross-species microarray hybridizations with targets derived from the *S. macrospora* WT, ΔSmtA-1, and ΔSmtA-2 strains on *N. crassa* 70-mer oligonucleotide arrays carrying 10,910 probes corresponding to the predicted 10,526 ORFs and to intergenic or telomeric regions of *N. crassa* (75).

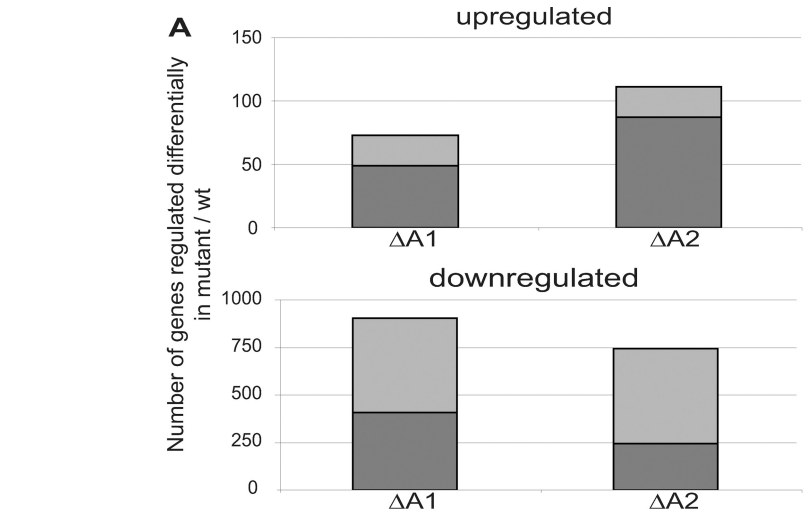
The targets were derived from *S. macrospora* mycelia grown for 4 to 5 days. At this stage, WT protoperithecia begin to develop into mature perithecia whereas the protoperithecia of ΔSmtA-2 do not develop any further. This stage of development had already been used to successfully compare the expression of the WT and other sterile mutant strains (e.g., pro mutants and the ΔSmta-1 mutant) by means of cross-species microarray analyses (55, 65).

Genes were defined as differentially expressed if they showed a >2-fold up- or downregulation with a P value of <0.05 in the mutant compared to the WT in both independent experiments (52). Compared to the WT, 73 genes were upregulated and 905 genes were downregulated in the ΔSmtA-1 strain and 111 genes were upregulated and 743 genes were downregulated in the ΔSmtA-2 strain. The genes that were regulated differentially in the ΔSmtA-1 and ΔSmtA-2 strains were further analyzed with the FunCat database (68). Genes with known or putative functions were sorted into 17 functional categories. In both mutants, the differentially regulated genes cover a broad range of functional categories not restricted to special physiological or metabolic functions (see Fig. S2 in the supplemental material). We found 311 genes to be significantly regulated only in the sterile mutant ΔSmtA-2 but not in ΔSmtA-1, suggesting that these genes might be involved directly or indirectly in sexual development processes (see Table S3 in the supplemental material). Interestingly, a large number of genes are regulated in ΔSmatA-1 and ΔSmtA-2 in the same way. In total, 23 genes were comparably upregulated and 497 were downregulated in both mutants (see Table S4 in the supplemental material) (Fig. 4).

To confirm the results of the microarray experiments, we analyzed the expression levels of different genes in the ΔSmtA-1 and ΔSmtA-2 mutants by quantitative real-time PCR. First, we analyzed the expression levels of four different genes involved in melanin biosynthesis: a polyketide synthase gene (*pks*), a scytalone dehydratase gene (*sdh*), a tetrahydroxynaphthalene reductase gene (*teh*), and a trihydroxynaphthalene reductase gene (*tih*) (22). In comparison to the WT, none of these genes was differentially regulated in ΔSmtA-1 whereas *sdh*, *the*, and *tih* are significantly downregulated in ΔSmtA-2 (see Fig. S3 in the supplemental material). The polyketide synthase gene (*pks*) was not affected in either mutant. We also analyzed two genes involved in copper homeostasis, *sod-2* and *ctr-3* (7). Both genes were significantly downregulated in fertile ΔSmtA-1, as well as in sterile ΔSmtA-2, but downregulation was stronger in ΔSmtA-2 (see Fig. S3 in the supplemental material). Furthermore, we checked the expression levels of the gene encoding a subunit of the origin recognition complex (*orc-3*) involved in cell proliferation (3). This gene was exclusively downregulated in the *SmtA-2* mutant (Fig. 4B). The expression level of *orc-3* was about 5-fold downregulated in ΔSmtA-2 but not in ΔSmtA-1 (see Fig. S3 in the supplemental material). For all three genes, *sod-2*, *ctr-3*, and *orc-3*, these results were in accordance with the microarray results.

DISCUSSION

A PPF domain protein but not an α domain protein is required for sexual reproduction in *S. macrospora*. The formation of fruiting bodies in homothallic *S. macrospora* is an apandrous process and lacks the interaction of two strains of op-



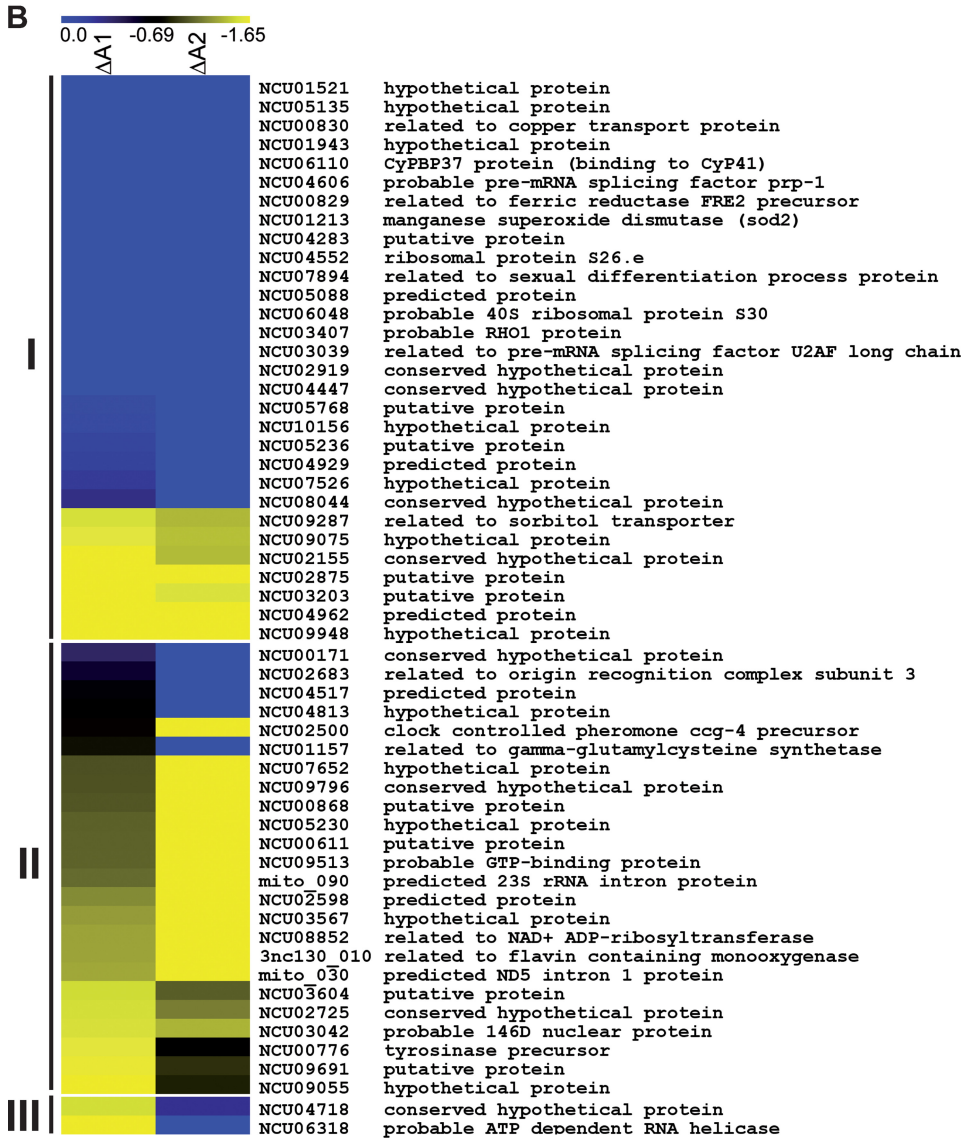


FIG. 4. Cross-species microarray analysis. (A) Numbers of genes that were found in cross-species microarray experiments to be up- or downregulated in ΔSmtA-1 and ΔSmtA-2, respectively. Genes that were found to be regulated the same way in both mutants compared to the WT are dark gray; genes that are regulated solely in one mutant are light gray. (B) Comparison of 60 genes that are the most strongly regulated in at least one mutant. According to the diagram, 30 are regulated the same way in both mutants (I), 24 are regulated exclusively in one mutant (II), and 2 are regulated the opposite way (III). Analysis was done with MultiExperimentViewer (MeV) (69).

posite mating types. Similar to other homothallic ascomycetes, the mating-type locus of *S. macrospora* contains counterparts of the sordariomycete *MAT* genes *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*, and *MAT1-2-1* (44, 59) (Fig. 1). Because the four homothallic *Neurospora* species *N. africana*, *N. dodgei*, *N. galapagosensis*, and *N. lineolata* contain only *MAT1-1*-specific genes and no sequences homologous to *MAT1-2* (29, 30) (Fig. 1), it has been predicted that the minimal mating-type structure of homothallic sordariomycetes should be *MAT1-1-1*, *MAT1-1-2*, and at least one HMG-encoding gene (either *MAT1-2-1* or *MAT1-1-3*) (17). *A. nidulans*, the best-studied species of homothallic *Eurotiomycetes*, carries two unlinked counterparts of *MAT1-1-1* (*SmtA-1*) and *MAT1-2-1* (*Smta-1*) (26) in its genome (Fig. 1), and both genes were shown to be involved in sexual development (57, 67).

Although SMTA-1 (MAT1-1-1) and SMTa-1 (MAT1-2-1) have been shown to interact physically (37), we demonstrate here that deletion of the α domain gene *SmtA-1* does not affect sexual reproduction in *S. macrospora*, in contrast to the deletion of HMG domain gene *Smta-1* (65). To the best of our knowledge, this is the first report on an ascomycete that carries an α domain gene at its *MAT* locus but does not require the protein for sexual development. Our result implies that *S. macrospora* is apparently able to complete the sexual cycle with only one HMG domain protein.

In this, *S. macrospora* resembles the mating-type situation of a group of fungi that diverged early, the *Zygomycota*. Here, it was recently demonstrated that *MAT* loci contain solely HMG-type genes (36) (Fig. 1). It was therefore proposed that an HMG domain-encoding gene was the ancestral state of the *MAT* locus for both heterothallic *Zygomycota* and the more highly evolved *Ascomycota* and *Basidiomycota*. The latter acquired α domain genes and homeodomain genes during evolution (10, 19, 36). The results shown here suggest that in homothallic *S. macrospora* only the ancestral HMG domain gene retained its function in sexual development while the conserved α domain gene is no longer needed for reproduction.

A *MAT1-1-2* gene encoding a PPF domain protein is consistently present in all *Sordariomycetes*, but no homolog has been identified outside this taxon. Here we show that *S. macrospora SmtA-2* (*MAT1-1-2*) plays an essential role in sexual development and reproduction. Like MAT1-1-2 proteins from other *Sordariomycetes*, SMTA-2 contains a conserved motif that has been termed the PPF domain (16, 17). Overall, however, MAT1-1-2 proteins are not well conserved. *S. macrospora* SMTA-2 shows only 72.1% and 22.9% identity to MAT1-1-2 of *N. crassa* and *P. anserina*, respectively. Therefore, it is not surprising that the mutant phenotype of ΔSmtA-2 can be complemented only by the *S. macrospora SmtA-2* gene and not by its *N. crassa* or *P. anserina* homolog (data not shown).

The sterile phenotype of ΔSmtA-2 resembles that of Δ*MAT1* mutants of homothallic *Gibberella zeae*. Deletion of three *MAT1-1*-specific mating-type genes rendered *G. zeae* self-sterile. However, because all three *MAT1-1*-specific genes, including the *SmtA-2* orthologue, were deleted it cannot be determined which gene causes the self-sterility of a Δ*MAT1-1* mutant *G. zeae* strain (45).

Interestingly, *G. zeae* ΔMAT1-1 (deletion of all *MAT1-1*-specific genes) and ΔMAT1-2 (deletion of *MAT1-2-1*) mutants were able to outcross to a self-fertile WT strain and both deletion strains can be crossed with each other in a heterothallic manner (45). When *S. macrospora* ΔSmta-1, ΔSmtA-2, or ΔSmtA-1 was crossed with a self-fertile spore color mutant, mature perithecia were formed at the intersection between the two parents. In each of those crosses, the spore color marker and the hygromycin resistance segregated in a 1:1 ratio within the progeny analyzed. Crossing of sterile ΔSmta-1 and fertile ΔSmtA-1 resulted in perithecia and ascospores, but crossing of the sterile ΔSmta-1 strain and the sterile ΔSmtA-2 strain did not result in perithecium formation. It is therefore possible that the two deletions affect a single function or pathway (32). Either both proteins are essential for hyphal fusion and deletion of both prevents heterokaryon formation, or both genes must be present in the same nucleus to be functional. Interestingly, nucleus-restricted expression of mating-type genes was demonstrated in *P. anserina* for the HMG domain gene *MAT1-1-3* (*SMR2*) (77).

Given that SMTa-1 is one of the main regulators of mating and might be involved in the orchestration of internuclear recognition and nuclear migration, similar to its counterpart FPR1 (MAT1-2-1) in *P. anserina*, it could also be considered to induce developmental arrest at this stage (77). In this case, it could be that SMTA-2 has to be present in the same nucleus to override the developmental block induced by SMTa1. In a combination where the two proteins are present in different nuclei, the developmental arrest might not be overcome (Fig. 5).

No phenotype was detected in a ΔSmtA-3 strain, and all phenotypic differences from the WT in a ΔSmtA-2/3 double-deletion mutant were shown to be caused by the deletion of *SmtA-2* alone; therefore, *SmtA-3* seems to encode a protein not essential for sexual reproduction. However, the gene might have regulatory functions, maybe as a kind of *cis*-regulatory element, associated with coexpression with *Smta-1* (61).

Expression of the pheromone precursor genes is controlled by the *MAT1-1*-specific mating-type proteins SMTA-1 and SMTA-2. In ascomycetes, two types of pheromone precursor genes and receptor genes are present in the same genome and it has been shown that expression of these genes in the yeast *S. cerevisiae* is regulated directly by the mating-type proteins (33). Similarly, heterothallic filamentous ascomycetes such as *N. crassa* and *P. anserina* express pheromone genes in a mating-type-dependent manner. Furthermore, pheromone genes are essential for male fertility in heterothallic ascomycetes (6, 14, 40). In *P. anserina*, mating-type genes activate their specific pheromone gene and repress the complementary pheromone gene (14). In contrast, transcription of pheromone receptor genes was shown to be mating type independent in heterothallic *N. crassa* (63). However, both pheromone genes and their cognate receptor genes are constitutively expressed in *S. macrospora* and in other homothallic ascomycetes (41, 57, 60, 63). In an earlier study, we showed that single-pheromone and -receptor mutant strains are not impaired in vegetative or sexual development. However, double-deletion strains lacking any compatible pheromone-receptor pair (Δpre2/ppg2 or Δpre1/ppg1) and the double-pheromone mutant (Δppg1/ppg2) display a drastically reduced number of perithecia and sexual spores, whereas deletion of both receptors genes (Δpre1/pre2) completely eliminates fruiting-body and ascospore formation

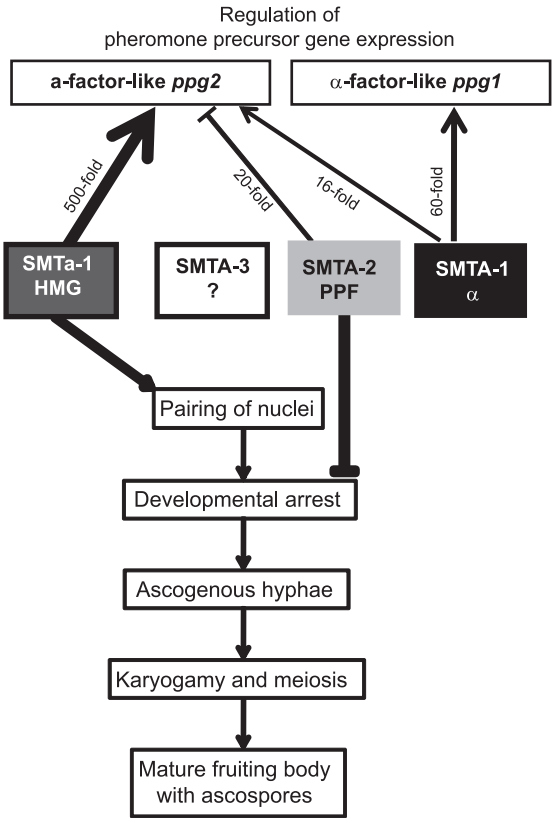


FIG. 5. Predicted model of the regulation of sexual development and pheromone gene expression in *S. macrospora* by mating-type proteins. The HMG domain protein SMTa-1 is essential for the pairing of nuclei prior to the formation of dikaryotic ascogenous hyphae and induces a developmental arrest which is overcome by the PPF domain protein SMTA-2. SMTA-3 and SMTA-1 are not involved in this process. However, the α domain protein SMTA-1 retains some function in the regulation of pheromone precursor genes. Positive regulation is shown as an arrow, and negative regulation is shown as a bar.

(48). This suggests that, in the absence of one of the two expressed pheromone-receptor pairs, the remaining pheromone-receptor pair can compensate for the loss of the other. Here, we show that in ΔSmtA-1 pheromone genes, *ppg1* and *ppg2* are 60-fold and 16-fold downregulated, respectively, while the expression of receptor genes was not significantly changed. The rather strong downregulation of both pheromone genes does not in any way impair sexual reproduction in ΔSmtA-1. Thus, the expression of *ppg1* and *ppg2* may still be above a threshold that is sufficient for normal fruiting-body and ascospore production. This is consistent with the previous finding that the deletion of both pheromone genes leads to a reduction in perithecium formation but not to complete sterility, suggesting that *S. macrospora* can bypass the need for pheromones but not for the corresponding receptors for sexual development (48).

In contrast to ΔSmtA-1, the *S. macrospora* ΔSmta-1 mutant lacking the *MAT1-2*-specific HMG transcription factor SMTa-1 displayed only a drastic reduction of *ppg2* lipopeptide gene expression, whereas the peptide pheromone gene *ppg1* was not affected (Fig. 3 and 5). This indicates that SMTa-1 has only a strong direct or indirect impact on the activation of *ppg2* gene expression (65) and the α domain protein SMTA-1, to a lesser extent, directly or indirectly activates both pheromone genes. Previously, two-hybrid analyses and *in vitro* assays revealed that SMTa-1 and SMTA-1 are capable of physically interacting (37). Thus, our data suggest that a heterodimer of SMTa-1 and SMTA-1 is involved in the activation of *ppg2* expression, whereas only SMTA-1 is required for *ppg1* expression.

In the sterile ΔSmtA-2 mutant, *ppg2* is upregulated 20-fold (Fig. 3). However, the increase in *ppg2* mRNA levels in ΔSmtA-2 alone seems not to be responsible for the sterile phenotype since overexpression of *ppg2* in the WT does not result in sterility (data not shown). In homothallic *G. zeae*, the *GzPPG1* transcript was not detected in strains with the *MAT1-1*-specific or *MAT1-2*-specific genes deleted. However, the *GzPPG2* transcript increased in the ΔMAT1 strain (41). Because all three *MAT1-1*-specific genes have been deleted in the ΔMAT1 strain, it is not clear whether upregulation of *GzPPG2* was caused by the deletion of *MAT1-1-1*, *MAT1-1-2*, or *MAT1-1-3* (45). In homothallic *A. nidulans*, deletion of *MAT1-1-1* has no effect on the expression of the peptide pheromone gene *ppgA* or the pheromone receptor genes (57). Taken together, the stringent mating-type-specific regulation of pheromone genes present in heterothallic filamentous ascomycetes seems be more relaxed in homothallic ascomycetes.

The mating-type proteins SMTA-1 and SMTA-2 affect the expression of several genes, including a common set of genes. We used a cross-species microarray analysis to examine the role of the *MAT1-1*-specific mating-type proteins SMTA-1 and SMTA-2 in the regulation of genes other than pheromone and receptor genes. In the ΔSmtA-1 and ΔSmtA-2 mutants, 978 and 853 genes, respectively, showed at least 2-fold alterations in mRNA abundance ($P < 0.05$). The majority of the genes are downregulated in both mutants. Only 7.5% (ΔSmtA-1) and 13.0% (ΔSmtA-2) of the differentially regulated genes are upregulated. In *S. cerevisiae*, the SMTA-1 ortholog α1, together with the transcription factors Mcm1 and Ste12 (8, 79), activates the expression of five *MAT*α-specific genes: the pheromone genes *Mfa1* and *Mfa2*, the a-factor receptor gene *STE3*, the agglutinin gene *SAG1*, and *YLR040C* (a gene of unknown function) (27). In *S. macrospora*, SMTA-1 interacts with the transcription factors MCM1 and STE12 as well (50, 51), but a much greater number of genes appears to be regulated by SMTA-1 than by α1 in *S. cerevisiae*. However, in contrast to the yeast study, our microarrays recorded directly and indirectly regulated genes.

The SMTA-2 protein contains no domain indicative of a transcription factor and does not interact with the putative transcription factor SMTA-1 or SMTa-1 or with the associated transcription factor MCM1 or STE12 (37, 50, 51); however, a large number of genes were also differentially regulated in the ΔSmtA-2 mutant. Interestingly, 519 genes were deregulated in both ΔSmtA-1 and ΔSmtA-2, indicating that both proteins, despite most probably not interacting directly, are involved in the same metabolic or developmental processes.

We performed quantitative real-time PCR experiments to analyze the expression of different sets of genes that are known to be involved in sexual differentiation processes. For all of the genes analyzed, these results were in accordance with the microarray results.

Not surprisingly, our cross-species microarray experiment

revealed that a few genes are regulated in ΔSmtA-2 as in all other sterile *S. macrospora* mutants (55, 65). As shown in Table S5 in the supplemental material, six genes are similarly downregulated in all of the sterile *S. macrospora* mutants that have been analyzed so far. These include the melanin biosynthesis gene *tih* (see Fig. S2 in the supplemental material) and the *app1* gene encoding the abundant perithecial protein APP, which has been shown to be expressed exclusively at late developmental stages and not in sterile mutants (54). Two of the genes regulated in all sterile *S. macrospora* mutants were also downregulated in ΔSmtA-1 (see Table S5 in the supplemental material). Overall, expression of this set of deregulated genes appears to be fruiting-body development specific rather than mating-type protein specific.

Model of a regulation network of the mating-type proteins and other, related factors in *S. macrospora*. This study revealed that the α domain-encoding gene *SmtA-1* (*MAT1-1-1*) is not required for sexual reproduction in the homothallic ascomycete *S. macrospora*. However, real-time PCR experiments showed that SMTA-1 has retained some regulatory functions in the regulation of pheromone genes (Fig. 5). The protein SMTA-1 has been shown to interact with other factors involved in mating, like STE12, MCM1, or the mating-type protein SMTa-1 (50, 51). This may indicate an evolutionary change from a key regulator of sexual development to a nonessential factor in *S. macrospora*.

The mating-type protein SMTA-3 also has no function in sexual reproduction. It lacks the functional HMG domain of MAT1-1-3 proteins from other *Sordariomycetes*. Since *SmtA-3* is cotranscribed with *Smta-1*, a putative regulatory function of *SmtA-3* as a *cis* element for the expression of *Smta-1* cannot be ruled out (61).

In contrast to *SmtA-1* and *SmtA-3*, *SmtA-2* is essential for sexual reproduction. The molecular function of SMTA-2 (MAT1-1-2) homologs in other members of the class *Sordariomycetes* is still unknown. However, its biological function was thoroughly analyzed in *P. anserina*. Mutations in PaMAT1-1-2 (SMR1) result in a complete arrest of sexual development after fertilization and before the formation of ascogenous hyphae. Therefore, Debuchy et al. (16) suggested that internuclear recognition preceding ascogenous hypha formation is associated with a developmental arrest that is overcome by the action of PaMAT1-1-2 (16). The sterile phenotype of ΔSmtA-2 implies that this function of SMTA-2 may be conserved in *S. macrospora* (Fig. 5). As has been shown previously, the HMG domain protein SMTa-1 is essential for fruiting-body and ascospore formation in *S. macrospora* (65). In heterothallic members of the class *Sordariomycetes*, the *MAT1-2*-specific HMG domain protein (MAT1-2-1) is supposed to control features specific to the *MAT1-2* mating type and to direct recognition between *MAT1-1* and *MAT1-2* nuclei prior to the formation of ascogenous hyphae (16, 77). In *P. anserina*, MAT1-2 strains expressing mutated versions of *PaMAT1-2-1* (*FPR1*) are weakly self-compatible and this suggests that mutations in *PaMAT1-2-1* allow the MAT1-2 nuclei to self-recognize (16, 77). It may well be, therefore, that during evolution, the HMG domain gene *Smta-1* (*MAT1-2-1*) of homothallic *S. macrospora* has accumulated mutations allowing self-recognition and therefore making the function of SMTA-1 (MAT1-1-1) superfluous. This, in turn, would also mean that deletion of *Smta-1* blocks nuclear recognition and therefore the formation of ascogenous hyphae und fruiting bodies (Fig. 5).

In contrast to *S. macrospora*, homothallic *A. nidulans* requires the α domain gene *MAT1-1-1* for sexual reproduction (57); however, a PPF domain gene is not present in the *A. nidulans* mating-type locus or elsewhere in the genome. Thus, our results suggest that the minimal mating-type structure of homothallic ascomycetes is at least one HMG gene and one other gene, either an α domain gene (*MAT1-1-1*) or a PPF domain gene (*MAT1-1-2*) (17).

In conclusion, our study revealed that the α domain protein SMTA-1 (MAT1-1-1) is not required for sexual reproduction of the homothallic ascomycete *S. macrospora*. Instead, we were able to demonstrate that the PPF domain protein SMTA-2 plays a key role in the regulation of sexual reproduction. It remains unclear whether the regulatory function of *SmtA-1* has been lost or whether it was replaced by other factors like *Smta-1* or even *SmtA-2*. Together, mating-type proteins of homothallic *S. macrospora* appear to control sexual reproduction by regulating a variety of essential cellular processes. Further studies are necessary to elucidate the molecular mechanism underlying this complex regulatory network.

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