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Appl. Environ. Microbiol. **2008, 74(19):6006. DOI: 10.1128/AEM.01188-08.**
Published Ahead of Print 8 August 2008.

Applied and Environmental Microbiology, Oct. 2008, p. 6006–6016 Vol. 74, No. 19
0099-2240/08/$08.00+0 doi:10.1128/AEM.01188-08

Asexual Cephalosporin C Producer *Acremonium chrysogenum* Carries a Functional Mating Type Locus▽

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Received 28 May 2008/Accepted 30 July 2008

***Acremonium chrysogenum*, the fungal producer of the pharmaceutically relevant β-lactam antibiotic cephalosporin C, is classified as asexual because no direct observation of mating or meiosis has yet been reported. To assess the potential of *A. chrysogenum* for sexual reproduction, we screened an expressed sequence tag library from *A. chrysogenum* for the expression of mating type (*MAT*) genes, which are the key regulators of sexual reproduction. We identified two putative mating type genes that are homologues of the α-box domain gene, *MAT1-1-1* and *MAT1-1-2*, encoding an HPG domain protein defined by the presence of the three invariant amino acids histidine, proline, and glycine. In addition, cDNAs encoding a putative pheromone receptor and pheromone-processing enzymes, as well as components of a pheromone response pathway, were found. Moreover, the entire *A. chrysogenum MAT1-1* (Ac*MAT1-1*) gene and regions flanking the *MAT* region were obtained from a genomic cosmid library, and sequence analysis revealed that in addition to Ac*MAT1-1-1* and Ac*MAT1-1-2*, the Ac*MAT1-1* locus comprises a third mating type gene, Ac*MAT1-1-3*, encoding a high-mobility-group domain protein. The α-box domain sequence of Ac*MAT1-1-1* was used to determine the phylogenetic relationships of *A. chrysogenum* to other ascomycetes. To determine the functionality of the Ac*MAT1-1* locus, the entire *MAT* locus was transferred into a *MAT* deletion strain of the heterothallic ascomycete *Podospora anserina* (the PaΔMAT strain). After fertilization with a *P. anserina* MAT1-2 (MAT$^+$) strain, the corresponding transformants developed fruiting bodies with mature ascospores. Thus, the results of our functional analysis of the Ac*MAT1-1* locus provide strong evidence to hypothesize a sexual cycle in *A. chrysogenum*.**

Acremonium chrysogenum (formerly named *Cephalosporium acremonium*) (22) is the industrial producer of the pharmaceutically relevant β-lactam antibiotic cephalosporin C (64). *A. chrysogenum* was isolated from seawater close to a sewage outfall area at Cagliari (Sardinia, Italy) in 1945 by Giuseppe Brotzu (6) and was found to produce, among other antibiotics, a β-lactam compound designated cephalosporin C, which is structurally related to penicillin (1). Today, cephalosporin derivatives are widely used in the treatment of infectious diseases and are one of the world's major biotechnological products, with a total world market of about $10 billion (5). The biosynthetic pathway for cephalosporin C is well characterized both genetically and biochemically and involves eight enzymatic steps that are catalyzed by seven different enzymes (38, 64). In addition, the expression of the corresponding biosynthesis genes is known to be controlled by several different transcriptional regulators (16, 63–66).

The genus *Acremonium* is a highly polyphyletic taxon containing distantly related fungi, including species associated with at least three or four ascomycete orders. Phylogenetic analyses revealed previously that *A. chrysogenum* shows affiliation with the order Hypocreales (25). To date, *A. chrysogenum* propagation has been accepted to be strictly asexual, by the formation of conidiospores and arthrospores (27, 42), and no associated teleomorph showing sexual propagation has been found. Thus, strains for improved industrial cephalosporin production can be obtained only by conventional mutagenesis and selection procedures or by molecular genetic techniques (29, 60, 72). However, the ability to mate fungi under controlled laboratory conditions would be a valuable tool for genetic analysis, as well as for conventional strain improvement programs (52).

In fungi, sexual reproduction is regulated by a genomic region referred to as the mating type (*MAT*) locus (20). In self-sterile (heterothallic) species, mating occurs between morphologically identical partners that are distinguished only by their *MAT* loci. The *MAT* locus consists of two dissimilar DNA sequences in the mating partners, termed the *MAT1-1* and *MAT1-2* idiomorphs (71). The *MAT1-1* locus invariably contains the *MAT1-1-1* gene, encoding a protein with an α-box domain. In most of the sordariomycetes, two other genes are also located in the *MAT1-1* locus: (i) the *MAT1-1-2* gene, encoding a protein with an HPG domain haboring the three invariant residues *h*istidine, *p*roline, and *g*lycine, and (ii) the *MAT1-1-3* gene, encoding a protein with a high-mobility-group (HMG) domain as a DNA-binding motif. The hallmark of the *MAT1-2* locus is the *MAT1-2-1* gene, encoding a protein with an HMG domain. In addition to the *MAT1-2-1* gene, other genes may also be present at the *MAT1-2* locus (14, 15, 70). In contrast to the genomes of heterothallic species, the genomes of self-fertile (homothallic) filamentous ascomycetes

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▽ Published ahead of print on 8 August 2008.

contain genes indicative of both mating types that can be either linked or unlinked (21, 55, 61, 80).

Moreover, several asexual filamentous fungi have been reported previously to contain functional, constitutively transcribed *MAT* genes. Among these are plant and human pathogens like *Bipolaris sacchari* and *Aspergillus fumigatus*, as well as biotechnologically relevant fungi such as *Penicillium chrysogenum* and *Aspergillus oryzae* (3, 17, 21, 26, 32, 49, 50, 67, 76). Analyses of the genome sequences of the asexual human pathogens *Aspergillus fumigatus* and *Penicillium marneffei* and that of *Penicillium chrysogenum* revealed the presence of transcriptionally active genes associated with sexual reproduction, including genes for pheromone production and recognition (21, 26, 49, 50, 76). These reports indicate that the absence of sexual reproduction is not due to disruptive mutations within *MAT* genes or other sex-related genes.

Here, we provide the first direct evidence of an *A. chrysogenum MAT1-1* (Ac*MAT1-1*) locus with at least three transcriptionally expressed genes. Furthermore, we provide a functional analysis of this Ac*MAT1-1* locus, which induced the formation of fruiting bodies and ascospores in the heterothallic ascomycete *Podospora anserina*. Moreover, we identified the homologue of a pheromone receptor gene that is known to function in mating and signaling in sexually reproducing filamentous ascomycetes, as well as homologues of a putative pheromone response pathway and putative pheromone-processing enzymes. To the best of our knowledge, this is the first report of an industrially relevant filamentous ascomycete for which the functionality of *MAT* genes is demonstrated by expression in a heterologous host. Our findings therefore open up the intriguing possibility of inducing mating and sexual reproduction as alternative strain improvement strategies for *A. chrysogenum*.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* strain K-12 XL1-Blue was used for general plasmid construction and maintenance (7). A list of all *A. chrysogenum* isolates used, with source details, is provided in Table 1. All strains were routinely maintained at 27°C on solid complex culture medium as described previously (41). *P. anserina* strains expressing MAT1-1 (MAT^-) and MAT1-2 (MAT^+) were derived from homokaryotic spores of *P. anserina* wild-type strains (18) containing only one of the two mating types. The *P. anserina* MAT (PaMAT) deletion mutant used in this study, PaΔMAT, was generated by replacing the Pa*MAT1-2* idiomorph by the *ura5*$^+$ transformation marker (11). Crosses and maintenance of *P. anserina* strains were performed on standard cornmeal agar (BMM) at 27°C under constant light.

Isolation of the Ac*MAT1-1* locus. The expressed sequence tag (EST) library from *A. chrysogenum* (Sandoz GmbH, Kundl, Austria) was used to identify an *A. chrysogenum* homologue of the *MAT1-1-1* gene critical for mating in other ascomycetes. To clone the entire Ac*MAT1-1* locus, the sequences of EST clone AcEST.4.5311 containing the putative *MAT1-1-1* gene were used to design primer pair MAT1-f (5′-CGGCCTCTGAACGGCTTCATG-3′) and MAT1-r (5′-GTCGTCGTTCTCGACATTCCA-3′). These primers were used in a rapid screening procedure (54) to screen an indexed *A. chrysogenum* cosmid library (16). The screening resulted in the isolation of cosmid P2-G5, which was subsequently used for the subcloning of the entire Ac*MAT1-1* locus by standard protocols (62). The subcloning of hybridizing DNA fragments from cosmid clone P2-G5 led to the construction of derivatives of the plasmid pDrive (Qiagen, Germany). Both strands of plasmid inserts were sequenced with universal primers T7 and SP6 (MWG-Biotech, Germany). The completion of the sequence was achieved by the synthesis of internal sequencing primers corresponding to regions where coverage was insufficient. Finally, the obtained partial sequences were combined into a single consensus sequence covering, in total, 12,076 bp of the Ac*MAT1-1* locus and adjacent regions.

Generation of cosmid P2-G5-hph for transformation of *P. anserina*. The screening of an *A. chrysogenum* cosmid library led to the isolation of cosmid P2-G5 carrying the complete Ac*MAT1-1* locus. In order to use this cosmid for the direct transformation of the PaΔMAT strain, we randomly integrated the *hph* resistance gene under the control of the *trpC* promoter of *Aspergillus nidulans* by using an in vitro recombination approach as described previously (16). The recombinant cosmid P2-G5-hph was examined by PCR and Southern analyses for the integrity of the Ac*MAT1-1* locus.

TABLE 1. Characteristics of *A. chrysogenum* strains used in this study, including mating types, loci of isolation, and sources

Strain	Mating type	Origin or locus of isolation	Reference or source
ATCC 14553	*MAT1-1*	Seawater, Sardinia, Italy	American Type Culture Collection, Manassas, VA
A3/2	*MAT1-1*	Derivative of strain ATCC 14553 obtained by random mutagenesis	57
CBS144.62	*MAT1-1*	Soil, Maharashtra, India	CBS fungal database, The Netherlands
CBS401.65	*MAT1-1*	Unknown	CBS fungal database, The Netherlands
CBS148.55	*MAT1-1*	Material from cooling aggregate, Rotterdam, The Netherlands	CBS fungal database, The Netherlands
ATCC 20339	*MAT1-1*	Seawater, Sardinia, Italy	DSMZ, Germany

Transformation of *P. anserina*. For transformation procedures, the PaΔMAT strain was cultivated in static liquid complete medium (45) at 27°C under constant light. The preparation and transformation of *P. anserina* protoplasts were performed as described previously (47). Fungal protoplasts were transformed with 15 μg of cosmid DNA, and the selection of transformants occurred on solid BMM medium supplemented with 50 U of hygromycin B/ml. The segregation of antibiotic resistance phenotypes in the sexual crosses was scored on the same medium.

Preparation of nucleic acids and DNA hybridization. For DNA and RNA extraction, *A. chrysogenum* strains were cultivated in liquid complex culture medium at 27°C with (180-rpm) or without shaking and *P. anserina* strains were grown in static liquid complete medium (45). After growth, resulting mycelia were removed, flash frozen, and ground under liquid nitrogen prior to nucleic acid extraction. Fungal genomic DNA and RNA were extracted by phenol-chloroform and chloroform-isoamyl alcohol methods as described previously (46). Southern blotting was performed with GeneScreen hybridization transfer membrane according to the instructions of the manufacturer (PerkinElmer, Boston, MA). Filters were hybridized with [α-^{32}P]dCTP-labeled probes by using standard methods (62).

PCR and semiquantitative RT-PCR for expression analyses. For use in semiquantitative reverse transcription (RT)-PCR, cDNA was synthesized as described previously (46), with the following modifications: 2-μg aliquots of total RNA were treated with 3.5 U of DNase I according to the recommendations of the manufacturer (Invitrogen, Germany), and RT was performed with 400 U of SuperScript II (Invitrogen, Germany) and deoxynucleoside triphosphates at a concentration of 0.33 mM. As a control for successful DNase treatment, each RT step was carried out twice, once with and once without reverse transcriptase. All samples were used together with genomic DNA as templates for the expression analysis of the Pa*MAT* and Ac*MAT* genes with the following primers: (i) for the Ac*MAT1-1-1* gene, primers MAT1-f and MAT1-r; (ii) for the Ac*MAT1-1-2* gene, primers MAT2-f (5′-CCGAGTCGCTCAAGAATGGCAA-3′) and MAT2-r (5′-TCATGGAAGACGCAAGCATA-3′); (iii) for the Ac*MAT1-1-3* gene, primers MAT3-f (5′-GTGCCTGAGACATTCGAATGG-3′) and MAT3-r (5′-CGATC GTACCAGAACTGTCGCT-3′); (iv) for the Pa*MAT1-1-1* (*FMR1*) gene, primers FMR1-f (5′-CCAAGAAGAAGGTCAACGGTT-3′) and FMR1-r (5′-GGA ACTTGCGTGTTGATACAGAT-3′); and (v) for the Pa*MAT1-2-1* (*FPR1*) gene, primers FPR1-f (5′-CCCTAACGCCTACATTCTTTA-3′) and FPR1-r (5′-GC ACAGTCGACTCCATTGTTT-3′).

PCR and RT-PCR analyses were conducted using a total volume of 50 μl

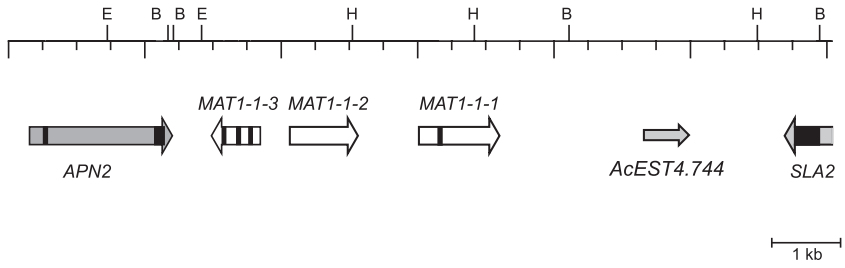

FIG. 1. Schematic illustration of the Ac*MAT1-1* locus and its flanking regions. The positioning and transcriptional directions of the mating type genes (Ac*MAT1-1-1*, Ac*MAT1-1-2*, and Ac*MAT1-1-3*) and the flanking genes (Ac*APN2* and Ac*SLA2*), as well as AcEST.4.744, are indicated by arrows. Introns are shown in black. E, EcoRI; B, BamHI; H, HindIII.

containing 150 μg of genomic DNA or 200 ng of cDNA, 10 pmol of each primer, 1 mM (each) deoxynucleoside triphosphates, and 5 U of HotMaster *Taq* polymerase (5 Prime, Germany). PCR conditions were as follows: 2 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 60 to 65°C, and 5 min at 68°C; and a final step of 15 min at 25°C.

Microscopy. For microscopic studies, *P. anserina* transformants were cultivated on solid BMM medium at 27°C under constant light. Morphologies of perithecia from *P. anserina* wild-type and transformant strains were analyzed using an Axiophot microscope (Carl Zeiss, Jena, Germany). Images were captured with an Axiovision digital system including the Zeiss Axiophot camera. Recorded pictures were processed with Adobe Photoshop CS2 software.

Sequence and phylogenetic analyses. The EST library from *A. chrysogenum* was used to identify *A. chrysogenum* homologues of genes critical for mating and pheromone signaling in other ascomycetes. Sequences of the cDNAs were obtained by custom sequencing carried out by Sandoz GmbH (Kundl, Austria). Fungal protein sequences from different sources were used as query sequences in searches with the basic local alignment search tool BLAST (2). For homology searching, the TBLASTN program was used. The E value cutoff used to select homologues was 1e−6. For validation of the identified *A. chrysogenum* genes, a bidirectional best-hit analysis was performed by using the DNA sequence of the identified *A. chrysogenum* EST clone as a query in a BLASTX search of the SwissProt/TrEMBL/TrEMBL_NEW database at the Swiss Institute of Bioinformatics (http://www.ch.embnet.org).

Protein sequence data were obtained from the NCBI Entrez public databases (http://www.ncbi.nlm.nih.gov/entrez/), and sequences were aligned using the ClustalX program (68). Phylogenetic analysis was carried out with programs contained in the program package PHYLIP version 3.6 (19). The same alignment was used for phylogenetic analysis performed by the distance matrix method and a maximum-parsimony method. Distance matrices were calculated using the program PROTDIST and were then used for constructing trees with the neighbor-joining program NEIGHBOR. The program PROTPARS was used for constructing maximum-parsimony trees. Statistical significance was evaluated by bootstrap analysis with 1,000 iterations of bootstrap samplings generated with SEQBOOT. A majority-rule consensus tree was subsequently generated using the program CONSENSE. The consensus trees were graphically displayed using TreeView (48).

RESULTS

Identification of EST clones coding for AcMAT proteins. To determine whether *MAT* genes are present in *A. chrysogenum*, we performed a BLAST analysis of the sequences present in an EST library from *A. chrysogenum* strain ATCC 14553. A TBLASTN search using the amino acid sequence corresponding to the *Neurospora crassa MAT1-1-1* (*MATA-1*) α-box domain gene as the query sequence revealed the presence of an 827-bp cDNA clone (AcEST.4.5311) encoding a polypeptide with 49% amino acid similarity to the *Neurospora crassa* MAT1-1-1 protein. A BLASTX search of the EMBL databases with the cDNA sequence of clone AcEST.4.5311 as the query showed similarity between the sequence of the putative *A. chrysogenum* α-box protein and those of α-box mating type proteins of other filamentous ascomycetes. The highest degrees of sequence similarity (with E values of 6e−41 and 3e−37) were those to MAT1-1-1 of the entomopathogenic fungus *Cordyceps takaomontana* (anamorphs, *Paecilomyces tenuipes* and *Isaria tenuipes*) (77–79) and MAT1-1-1 of the asexual fungal pathogen *Fusarium poae* (32). By using the sequence of the MAT1-1-2 protein of *Cordyceps takaomontana* as the query sequence for a TBLASTN search, the product of a 549-bp EST clone (AcEST.4.20371) was found to show the highest degree of sequence identity. A BLASTX search of the EMBL databases with the nucleotide sequence of clone AcEST.4.20371 revealed a high degree of similarity to the sequence encoding the MAT1-1-2 protein of *Claviceps purpurea* (78).

A TBLASTN search with MAT1-2-1 proteins from different filamentous ascomycetes failed to identify an EST clone encoding an HMG domain with significant similarity (data not shown). Thus, we concluded that *A. chrysogenum* ATCC 14553 is a MAT1-1 strain.

Cloning of the entire *MAT1-1* locus of *A. chrysogenum* ATCC 14553. To clone the entire Ac*MAT1-1* locus, the sequences of EST clone AcEST.4.5311 containing the putative Ac*MAT1-1-1* gene were used to design the primer pair MAT1-f/MAT1-r. Subsequent genomic PCR conducted with this primer pair produced a 336-bp amplicon, and DNA sequencing confirmed its identity (Fig. 1). The primers were then used in a rapid screening procedure (54) to screen an indexed *A. chrysogenum* cosmid library. The isolated clone P2-G5 was subsequently used for the subcloning of the entire Ac*MAT1-1* locus. The sequence of the subcloned DNA, covering in total 12,076 bp of the mating type locus and adjacent regions, was determined. Combined computer and RT-PCR analyses of this sequence resulted in the identification of three putative *MAT* genes which, according to the nomenclature of Turgeon and Yoder (71), were termed Ac*MAT1-1-1*, Ac*MAT1-1-2*, and Ac*MAT1-1-3* (Fig. 1).

The Ac*MAT1-1-1* open reading frame (ORF) encodes a polypeptide of 375 amino acids (aa) and contains a single intron of 55 bp, which habors typical *A. chrysogenum* splice sites (30). Comparison of the predicted amino acid sequence with known sequences from the databases revealed that the AcMAT1-1-1 protein is most similar to the MAT1-1-1 protein of *Gibberella zeae* (43.8% amino acid identity in a 226-aa overlap). The predicted DNA-binding motif of the AcMAT1-1-1 protein is very similar to the conserved α-box domain found in the *Saccharomyces cerevisiae* Matα1p protein and in MAT1-1-1 homologues from other Sordariaceae (Fig. 2A).

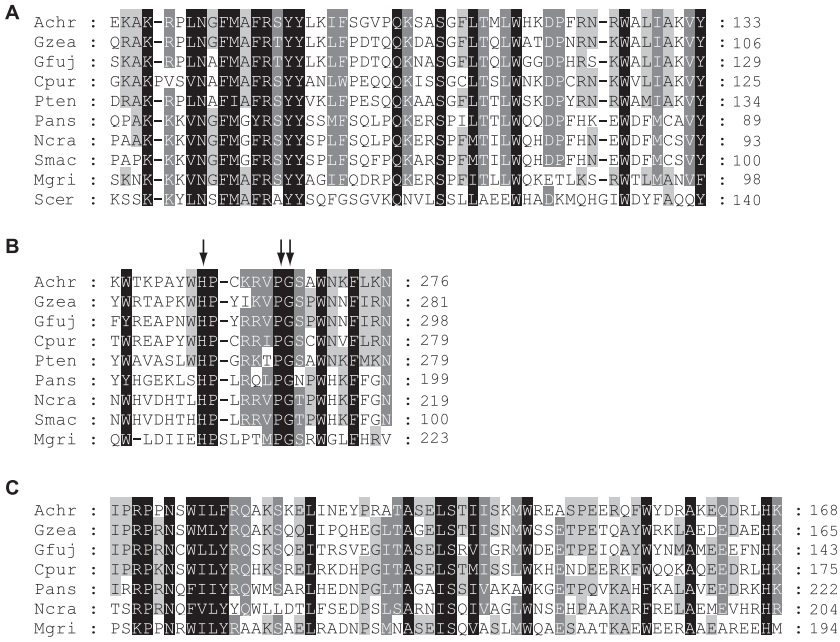

FIG. 2. Conserved domains of mating type proteins encoded by the Ac*MAT1* locus. (A) Multiple-sequence alignment of the α-box domain region of the AcMAT1-1-1 protein with α-box domain regions of proteins from other ascomycetes. Abbreviations and accession numbers corresponding to the proteins are as follows: Achr, *A. chrysogenum* (AM983455); Gzea, *Gibberella zeae* (Q9HEV7); Gfuj, *Gibberella fujikuroi* (O93925); Cpur, *Claviceps purpurea* (BAD72604.1); Pten, *Paecilomyces tenuipes* (BAC67541); Pans, *P. anserina* (P35692); Ncra, *Neurospora crassa* (P19392); Smac, *Sordaria macrospora* (O42837); Mgri, *Magnaporthe grisea* (BAC65091.1); and Scer, *S. cerevisiae* (YP_087100.1). Numbers to the right of the sequences indicate amino acid positions. Shading indicates the degrees of similarity. (B) Multiple-sequence alignment of the HPG domain of the AcMAT1-1-2 protein with HPG mating type proteins from other ascomycetes. The positions of the conserved amino acid residues His, Pro, and Gly are indicated by arrows. For abbreviations, see the legend to panel A. Accession numbers corresponding to the proteins are as follows: *A. chrysogenum*, AM983456; *Gibberella zeae*, AAG42811.1; *Gibberella fujikuroi*, AAC71054.1; *Claviceps purpurea*, BAD72603.1; Pten, BAC67540; *P. anserina*, S39889; *Neurospora crassa*, S65583; *Sordaria macrospora*, CAA71626.1; and *Magnaporthe grisea*, BAC65092.1. (C) Multiple-sequence alignment of the HMG domain of the AcMAT1-1-3 protein with MAT1-1-3 HMG mating type proteins from other ascomycetes. For abbreviations, see the legend to panel A. Accession numbers corresponding to the proteins are as follows: *A. chrysogenum*, AM983457; *Gibberella zeae*, Q9HEV9; *Gibberella fujikuroi*, AAC71053.1; *Claviceps purpurea*, BAD72602.2; *P. anserina*, Q08143; *Neurospora crassa*, S65584; and *Magnaporthe grisea*, BAC65093.2. Amino acid residues conserved in all species are indicated in white with black background shading. Residues conserved in all species with the exception of one or two are indicated in white with gray background shading. Residues conserved in the majority of species are indicated in black with gray background shading.

The Ac*MAT1-1-2* gene encodes a polypeptide of 331 aa and is not interrupted by introns. The AcMAT1-1-2 protein displayed the highest degree of identity (31.6% amino acid identity in a 335-aa overlap) to the MAT1-1-2 protein containing the conserved HPG domain of *Epichloe typhina*, an endophytic member of the Clavicipitaceae (Fig. 2B). The coding sequence proposed for the third gene, Ac*MAT1-1-3*, translates into a 190-aa protein and contains three introns of 46, 52, and 48 bp showing typical *A. chrysogenum* splice sites (30). Comparison of the predicted AcMAT1-1-3 amino acid sequence with known sequences from the databases revealed that AcMAT1-1-3 is most similar to the MAT1-1-3 protein of *Ephelis japonica* (Clavicipitaceae; 51.6% amino acid identity in a 182-aa overlap). The HMG domain DNA-binding motif is highly similar to HMG domains found in MAT1-1-3 homologues of other sordariomycetes (Fig. 2C). The analysis of the regions upstream and downstream of the mating type genes revealed the presence of an ORF similar to *S. cerevisiae APN2* encoding a DNA lyase protein (31), as well as a homologue of the *S. cerevisiae SLA2* gene encoding a cytoskeleton assembly protein (28). Frequently, both genes are found close to the *MAT* loci in filamentous ascomycetes (15, 17, 21, 61).

MAT genes are very useful for phylogenetic analysis due to their high evolution rate (53, 69, 73). We therefore performed an analysis based on the MAT1-1-1 α-box domain, in order to obtain further information about the phylogenetic position of *A. chrysogenum* among the ascomycetes. Identical phylogenetic trees were derived using neighbor-joining and parsimony analyses with the α-box domain of the *S. cerevisiae* Matα1p as the out-group. According to the cladogram shown in Fig. 3, *A. chrysogenum* shows affiliation with the order Hypocreales and is phylogenetically most closely related to *Metarhizium anisopliae*.

Transcriptional expression of mating type gene *MAT1-1-3*. Since EST clones of Ac*MAT1-1-1* and Ac*MAT1-1-2* were identified in the EST library, both genes appear to be transcriptionally expressed in *A. chrysogenum*. To determine whether Ac*MAT1-1-3*, the putative third *MAT* gene, was expressed, RNA was isolated from *A. chrysogenum* and cDNA was synthesized using Ac*MAT1-1-3*-specific primers. For comparison, cDNAs and amplicons using genomic DNA as the template were generated with primer pairs specific for Ac*MAT1-1-1*, Ac*MAT1-1-2*, and Ac*MAT1-1-3* (Fig. 4). Sequencing of the Ac*MAT1-1-3* cDNA confirmed the splicing of the three introns predicted from the genomic DNA, indicating that all three

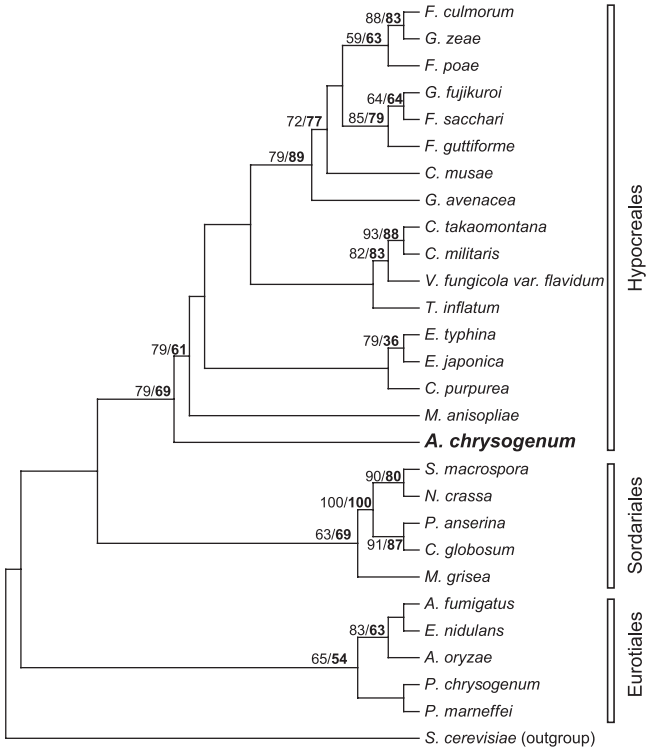

FIG. 3. Phylogenetic tree of MAT1-1-1 α-box domains generated by maximum-parsimony and neighbor-joining analyses. Fifty-four amino acid positions were included in the analyses. The tree shown is based on a consensus tree calculated with the PHYLIP programs PROTPARS and NEIGHBOR. The numbers (percentages) indicate the support based on 1,000 replications of the neighbor-joining analysis (numbers to the left of the slashes) and of the maximum-parsimony procedure (numbers to the right of the slashes). Accession numbers corresponding to the analyzed amino acid sequences are as follows: *A. chrysogenum*, AM983455; *Fusarium culmorum*, CAD59609.3; *Gibberella zeae*, A9CCT5; *Fusarium poae*, CAD59610.3; *Gibberella fujikuroi*, O93925; *Fusarium sacchari*, BAE94379.1; *Fusarium guttiforme*, AAQ18160; *Colletotrichum musae*, CAD59611.3; *Gibberella avenacea*, CAD59608.4; *Cordyceps takaomontana*, BAD95880.1; *Cordyceps militaris*, BAD72600.1; *Verticillium fungicola* var. *flavidum*, BAE93605.1; *Tolypocladium inflatum*, BAE93602.1; *Epichloe typhina*, BAD72612.1; *Ephelis japonica*, BAD72608.1; *Claviceps purpurea*, BAD72604.1; *Metarhizium anisopliae*, BAE93598.1; *Sordaria macrospora*, O42837; *Neurospora crassa*, P19392; *P. anserina*, P35692; *Chaetomium globosum*, XP_001222681.1; *Magnaporthe grisea*, BAC65091.1; *Aspergillus fumigatus*, AAX83123.1; *Emericella nidulans*, Q7Z896; *Aspergillus oryzae*, Q2U537; *Penicillium chrysogenum*, AM904544; *Penicillium marneffei*, Q1A3S7; and *S. cerevisiae*, YP_087100.1.

Ac*MAT* genes are transcriptionally active. In addition to the *MAT* genes, we identified an EST clone corresponding to the *APN2* gene; however, we did not identify an EST clone corresponding to the *SLA2* gene. The predicted intergenic region between Ac*MAT1-1-1* and the Ac*SLA2* gene appeared to be exceptionally large (Fig. 1). Therefore, we used the 4,184-bp sequence of this region in a BLASTN analysis of the *A. chrysogenum* EST library and identified the 662-bp EST clone AcEST.4.744 corresponding to a transcript of this region. However, the sequence of this transcript has no similarity to any protein or EST clone transcript in the databases.

Mating type loci in different *A. chrysogenum* isolates. To identify a putative *MAT1-2* locus and to analyze the distribution of the two mating types, five *A. chrysogenum* isolates were examined using the α-box domain sequence-specific Ac*MAT1-1-1* primers. Only a limited set of six isolates was available from culture collections, including strain ATCC 14553, originally isolated by Giuseppe Brotzu, and the derivative A3/2, which stems from an industrial production line (57). In addition, four other strains collected from at least two different geographic sites (Table 1) were used for our analysis. All strains most probably belong to the same taxon since they contain completely identical internal transcribed spacer sequences (data not shown). Notably, the ITS2 sequence is highly suitable for evaluating whether the taxa of a species can potentially interbreed and has already been used for this purpose for numerous eukaryotes (10). In all strains tested, a 336-bp DNA fragment specific for the Ac*MAT1-1-1* α-box mating type gene was present, leading to the conclusion that all *A. chrysogenum* isolates analyzed are either heterothallic *MAT1-1* strains or homothallic strains with a *MAT1-2* allele elsewhere in the genome or with only the *MAT1-1* locus.

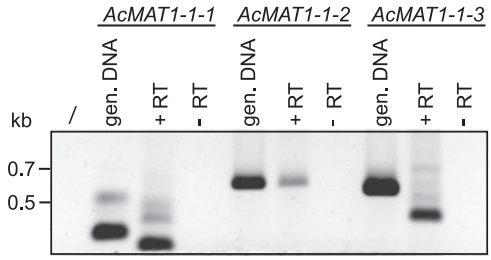

FIG. 4. Transcriptional expression of the genes Ac*MAT1-1-1*, Ac*MAT1-1-2*, and Ac*MAT1-1-3*. Primer pairs as indicated in Materials and Methods were used to generate cDNA or DNA fragments specific for the three ORFs of the Ac*MAT1-1* locus. RNA transcripts (cDNA) either with (+ RT) or without (− RT) reverse transcriptase were amplified by RT-PCR. gen. DNA, genomic DNA.

Functional analysis of the Ac*MAT1-1* locus in *P. anserina*. The failure to detect any AcMAT1-2 strains makes it impossible to conduct crossing experiments with strains of the opposite mating type. We therefore performed a functional analysis of the Ac*MAT1-1* locus with *P. anserina* as a heterologous host. This heterothallic ascomycete carries a *MAT1-1* locus with a high degree of sequence similarity to the Ac*MAT1-1* locus (Fig. 2). Hence, we transferred cosmid clone P2-G5-hph containing the entire Ac*MAT1-1* locus into a PaΔMAT strain (11), yielding the PaΔMAT-Ac*MAT1-1*^{ect} strain carrying ectopically expressed Ac*MAT1-1* (Fig. 5A), since it was shown recently that an ectopically expressed *MAT* locus in this deletion strain confers normal fertility in a cross (11, 51, 75). In contrast, a resident *MAT* locus was shown previously to interfere with extra copies of *MAT* locus genes, leading to ascospore lethality (13).

PCR and RT-PCR analyses confirmed that all three Ac*MAT1-1* genes were integrated and transcriptionally expressed in the *P. anserina* transformants (Fig. 5B and C). Three individual transformants carrying the transcriptionally expressed Ac*MAT1-1* locus were then analyzed further by fertilization with spermatia of a PaMAT1-2 (MAT$^+$) strain (Fig. 5A). All three developed further and generated numerous perithecia, indistinguishable from those produced in *P. anserina* wild-type matings, in crosses with the tester strain (Fig.

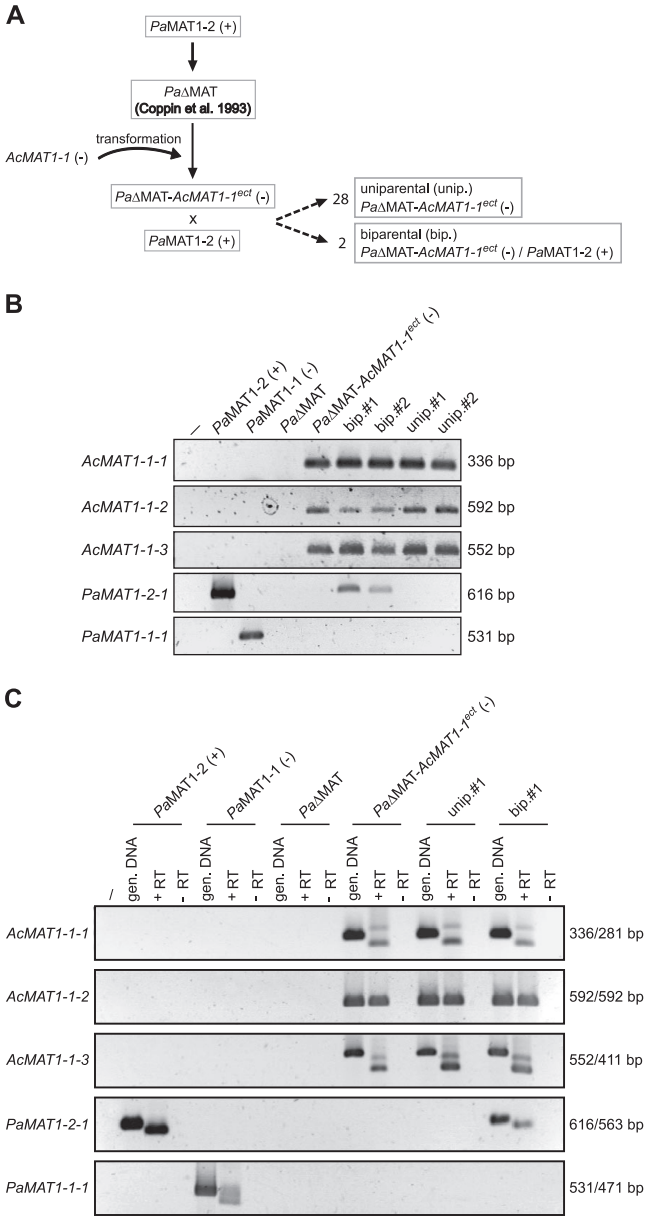

FIG. 5. Rescue of the function of the *P. anserina* mating type locus by the Ac*MAT1-1* locus. (A) Flow diagram depicting the origin of strains obtained from the mating (×) of a transformant PaΔMAT-Ac*MAT1-1*^{ect} (MAT$^-$) strain with the PaMAT1-2 (MAT$^+$) strain. (B) Molecular phenotypes of PaMAT1-2, PaMAT1-1, and PaΔMAT strains and of the transformant PaΔMAT-Ac*MAT1-1*^{ect} strain expressing the Ac*MAT1-1* locus, as well as of the biparental ascospore isolates (bip#1 and bip#2) and 2 of the 28 uniparental ascospore isolates (unip#1 and unip#2). (C) Transcriptional expression of the Ac*MAT1-1* locus, as well as the Pa*MAT1-2-1* (*FPR1*) and Pa*MAT1-1-1* (*FMR1*) genes, in *P. anserina* strains as indicated above the gel. +RT and − RT, with and without reverse transcriptase; gen. DNA, genomic DNA. The sizes of the DNAs and cDNA amplicons are the first and second values, respectively, given at the right margin.

6A). However, in most cases, ascus rosettes of perithecia comprised only 3 to 20 asci and the number of asci with fewer than four ascospores was significantly increased compared to the number of such asci produced by the wild type (Fig. 6B). *P. anserina* has a pseudohomothallic life cycle and develops four-spored asci, in which ascospores contain two nuclei, one of each mating type. A typical binucleate spore is a heterokaryon, i.e., it contains both mating types, and can therefore give rise to a self-fertile mycelium (Fig. 6C). Only a few asci contain five spores, three binucleate and two small uninucleate spores, which produce homokaryons.

To investigate the inheritance of the heterologous *MAT* locus, 30 randomly isolated ascospores of the fertilized PaΔMAT-Ac*MAT1-1*^{ect} strain were then tested for simultaneous expression of the endogenous Pa*MAT1-2-1* (*FPR1*) gene and the heterologous Ac*MAT1-1* genes (Fig. 5C). Only 2 of 30 transformants expressed the *MAT* genes from both loci, while the other 28 seemed to express only the Ac*MAT1-1* genes (Fig. 5A and C). Thus, these results demonstrate that the Ac*MAT1-1* locus is transcriptionally expressed and can drive sexual reproduction in *P. anserina*.

Identification of sex-related genes in *A. chrysogenum*. Knowing that Ac*MAT1-1* is able to carry out fertilization functions in *P. anserina*, which suggests the potential of *A. chrysogenum* for sexual reproduction, we then performed in silico analyses to identify sex-related genes in *A. chrysogenum*. Two different classes of pheromones are known to be involved in sexual reproduction in heterothallic as well as in homothallic filamentous ascomycetes. One class of genes encodes peptide pheromone precursors that contain multiple copies of the mature peptides flanked by protease cleavage sites, while the other class of pheromone genes encodes a small protein with a CAAX motif at the carboxy terminus. This motif is expected to produce a mature lipopeptide pheromone with a C-terminal carboxymethyl isoprenylated cysteine (12, 33, 39). Therefore, to identify putative pheromone precursors encoded by the *A. chrysogenum* genome, amino acid sequences of the peptide and a lipopeptide pheromone precursor of known fungal pheromone precursors were used as query sequences in TBLASTN searches. No significant hits were found. However, a cDNA encoding a putative pheromone receptor and EST clones encoding putative homologues of proteins involved in the processing of pheromone precursors in *S. cerevisiae* were identified in the EST library (Table 2).

In the yeast *S. cerevisiae*, after the binding of the pheromones to a cell type-specific receptor, the signal is transmitted by the interaction of a heterotrimeric G protein composed of Gα (Gpa1p), Gβ (Ste4p), and Gγ (Ste18p) through a downstream mitogen-activated protein (MAP) kinase cascade encoded by *STE20*, *STE11*, *STE7*, and *FUS3*. Homologues of all these genes, along with other conserved genes of the *S. cerevisiae* pheromone response pathway, except *FAR1*, *STE5*, *KSS1*, *DIG1*, and *DIG2*, were identified in the *A. chrysogenum* EST library (Table 3).

DISCUSSION

Filamentous ascomycetes lacking any sexual stages have traditionally been placed within the phylum Deuteromycota or have been termed Fungi Imperfecti. This artificial group includes plant and human pathogens, as well as biotechnologically important fungi, such as the β-lactam-producing fungi *Penicillium chrysogenum* and *A. chrysogenum*. Recently, however, there has been a proliferation of reports describing the detection of *MAT*

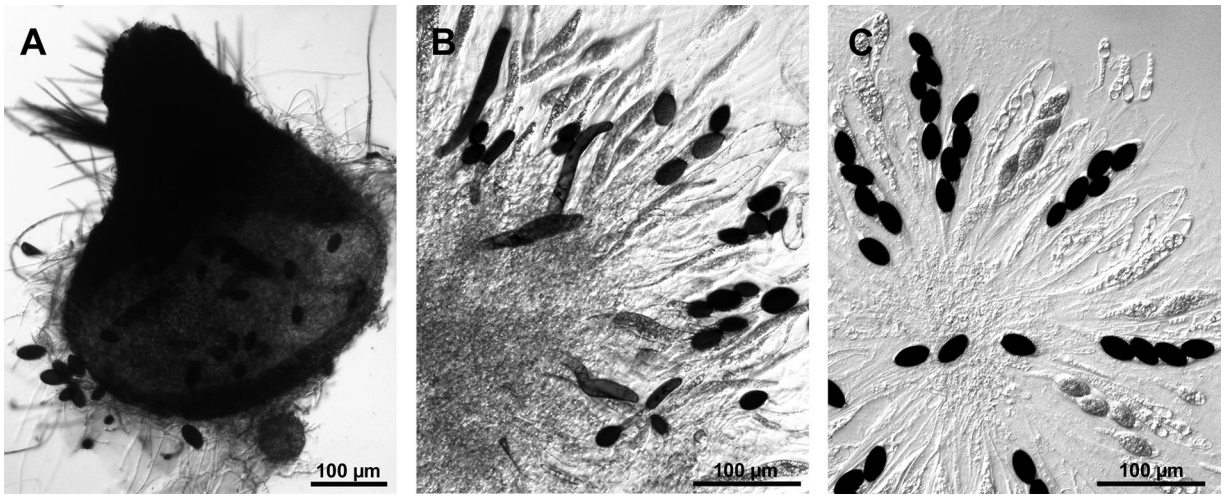

FIG. 6. Microscopic investigation of *P. anserina* strains expressing the Ac*MAT1-1* locus. (A) Perithecium from a strain obtained from the mating of PaΔMAT-Ac*MAT1-1*^{ect} (MAT$^-$) and PaMAT1-2 (MAT$^+$) strains. (B) Ascus rosette from the strain shown in panel A. (C) For comparison, an ascus rosette from a strain obtained from a PaMAT1-1 (MAT$^-$) and PaMAT1-2 (MAT$^+$) wild type cross.

genes and homologues of genes known to be required for sexual pheromone production within species of this group. Thus, there is accumulating evidence suggesting that an increasing number of Fungi Imperfecti have a cryptic sexual cycle and that asexual ascomycetes have the genetic machinery to mate and to develop fertile fruiting bodies, as well as to undergo a full meiotic sexual cycle (3, 21, 26, 32, 50, 67, 76).

***A. chrysogenum* has a *MAT1-1* mating type locus.** In this study, *A. chrysogenum* strains were shown to carry a *MAT1-1* locus structurally similar to *MAT* loci from heterothallic Sordariales and Hypocreales. The *MAT1-1* locus from *A. chrysogenum* contains at least three genes, Ac*MAT1-1-1*, Ac*MAT1-1-2*, and Ac*MAT1-1-3*, encoding proteins with conserved domains: an α-box domain, an HPG domain, and an HMG domain, respectively (Fig. 2).

Since we did not succeed in detecting an AcMAT1-2 strain, the exact extension of the *MAT1-1* locus cannot be defined. Recently, the sequences in close proximity to *MAT* loci from diverse ascomycetes were investigated, and it emerged that in an ancestral configuration, *MAT* loci lie between homologues of *S. cerevisiae APN2* and *SLA2* (9, 15). Similarly, in *A. chrysogenum*, *APN2* and *SLA2* homologues were identified downstream of the Ac*MAT1-1-3* and Ac*MAT1-1-1* genes, respectively (Fig. 1). An *APN2-SLA2* gene arrangement identical to that in *A. chrysogenum* is also present in *Neurospora crassa*, *P. anserina*, several *Gibberella* species, *Aspergillus fumigatus*, and *Neosartorya fischeri* (15, 21, 61). Hence, according to the configuration of the flanking genes, the Ac*MAT1-1* locus exhibits a rather ancestral structure.

The genus *Acremonium* is considered to be a highly polyphyletic genus containing distantly related species (25). Phylogenetic analyses based on partial sequences of the 18S rRNA revealed the affiliation of *A. chrysogenum* with the order Hypocreales (25). Likewise, the *MAT1-1-1* trees obtained from

TABLE 2. *A. chrysogenum* EST clones encoding putative pheromone-processing enzymes

S. cerevisiae protein	Function	E value[a]	Corresponding cDNA clone/length (bp)/accession no.	Closest homologue (GeneID no.) and E value[b]
Kex1p	Carboxypeptidase	8e−020	AcEST.4.7623/678/FM200412	*Gibberella zeae* hypothetical protein (FG10145.1), 4e−45
Ste13p	Dipeptidyl aminopeptidase	2e−005	AcEST.4.379/797 (may correspond to another dipeptidyl aminopeptidase)/ FM200421	*Gibberella zeae* hypothetical protein (FG01095.1), 2e−88
Ste24p	CAAX prenyl protease	2e−033	AcEST.4.3818/734/FM200424	*Gibberella zeae* hypothetical protein (FG0590.1), 2e−89
Ste23p	Secreted/periplasmic Zn-dependent peptidases	3e−048	AcEST.4.726/1,100/FM200423	*Gibberella zeae* hypothetical protein (FG06911.1), 1e−108
Ram1p	Prenyl transferase, β subunit	9e−006	AcEST.4.6556/681/FM200414	*Gibberella zeae* hypothetical protein (FG08771.1), 1e−23
Ram2p	Prenyl transferase, α subunit	2e−016	AcEST.4.17786/621/FM200415	*Gibberella zeae* hypothetical protein (FG05911.1), 8e−66
Ste6p	ABC-type multidrug transport system	2e−043	AcEST.4.3111/1,079/FM200417	*Gibberella zeae* hypothetical protein (FG06881.1), 1e−115

[a] Result from TBLASTN.
[b] Result from BLASTX.

TABLE 3. *A. chrysogenum* EST clones encoding putative components of a pheromone response pathway

S. cerevisiae protein(s)	Function	E value[a]	Corresponding cDNA clone/length (bp)/accession no.	Closest homologue (GeneID no.) and E value[b]
Ste2p	Peptide receptor	No hits		
Ste3p	Lipopeptide receptor	No hits	AcEST.4.6856/427[c]/FM200413	*Gibberella zeae* PRE1 (FG07270.1), 6e−012
Gpa1p	α subunit of G protein	8e−065	AcEST.4.13545/2,030/FM200411	*Fusarium oxysporum fga1* product (Q96VA7), 1e−179
Ste4p	β subunit of G protein	2e−074	AcEST.4.8282/1,535/FM200416	*Gibberella moniliformis* G protein β subunit (Q1PBD2), 0.0
Ste18p	γ subunit of G protein	3e−009	AcEST.4.18338/1,039/FM200422	*Magnaporthe grisea* G protein γ subunit (MGG1), 2e−31
Cdc24p	Guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases	1e−004	AcEST.4.7446/759/FM200408	*Gibberella zeae* hypothetical protein (FG10511.1), 9e−63
Cdc42p	Small Rho-like GTPase	8e−080	AcEST.4.22441/1,035/FM200409	*Coccidioides immitis* cell division control protein 42, 3e−54
Bem1p	Scaffold protein for complexes including Cdc24p, Ste5p, Ste20p, and Rsr1p	7e−020	AcEST.4.4166/660/FM200407	*Gibberella zeae* hypothetical protein (FG05261.1), 1e−47
Ste20p	p21-activated kinase	5e−032	AcEST.4.15304/1,283/FM200419	*Gibberella zeae* hypothetical protein (Q4IBC4), 1e−164
Ste11p	MEK kinase	8e−083	AcEST.4.15304/1,283/FM200419	*Gibberella zeae* hypothetical protein (Q4IBC4), 1e−164
Ste7p	MAP kinase kinase	2e−037	AcEST.4.10992/1,216/FM200418	*Gibberella zeae* hypothetical protein (FG07295.1), 1e−177
Fus3p	MAP kinase	4e−065	AcEST.4.17143/1,160/FM200410	*Gibberella zeae* MAP kinase hypothetical protein (MGV1 protein; FG10313.1), 1e−145
Ste12p	Homeobox transcription factor	No hits	AcEST.4.5637/1,324[c]/FM200420	*Gibberella zeae* transcription factor Fst12 (Q8NKD8), 1e−107
Kss1p	MAP kinase	No hits		
Ste5p	Scaffold protein	No hits		
Far1p	Cyclin-dependent kinase inhibitor	No hits		
Dig1p, Dig2p	Regulatory proteins required for MAP kinase-imposed repression	No hits		

[a] Result from TBLASTN.
[b] Result from BLASTX.
[c] STE2 was selected by the homologous protein of *Gibberella zeae*.

maximum-parsimony and neighbor-joining analyses show a common branching pattern for *A. chrysogenum* and species of the order Hypocreales and a close relationship to species of the family Clavicipitaceae (Fig. 3). Clavicipitaceous fungi and related anamorphs contain diverse econutritional groups such as pathogens of plants, insects, nematodes, and fungi, as well as endophytes of plants and saprophytes. Divergent groups of the Clavicipitaceae, including the species *Cordyceps takaomontana* and *Cordyceps militaris*, are known to lack the mating type gene *MAT1-1-3*, while more ancient members of this family, e.g., *Claviceps purpurea* and *Metarhizium anisopliae*, possess a *MAT1-1-3* gene (77). Given that the *MAT1-1* locus of *A. chrysogenum* contains a *MAT1-1-3* homologue, it may be considered to be a phylogenetically old member of the Clavicipitaceae family.

In addition to the three conserved *MAT* genes, a fourth putative ORF corresponding to a 662-bp EST clone, AcEST.4.744, was identified between Ac*MAT1-1-1* and *SLA2*. In *Neurospora crassa*, two genes encoding anonymous transcripts (*eat*) with mating type-specific size differences were identified in the region surrounding the *MAT* idiomorphs. One of these genes, *eat-2*, encodes a protein with a domain highly similar to a domain present in plasma membrane ATPases (58). However, the sequence of AcEST.4.744 bears no similarity to that of either *Neurospora crassa eat-2* or *eat-1* or to that of any other EST clone in the databases. Due to the lack of an AcMAT1-2 strain, mating type-specific transcript differences could not be analyzed.

Based on the fact that we identified only MAT1-1 strains of *A. chrysogenum*, we cannot rule out that a MAT1-2 strain does exist. The failure to identify it may be due to the small sample size used in this study. However, at this stage we cannot decide whether *A. chrysogenum* has a heterothallic or homothallic lifestyle. There are four ways fungi can be homothallic: (i) they can harbor a fused *MAT* locus comprising both idiomorphs, (ii) they can harbor both *MAT* alleles at different loci in the genome, (iii) they can switch the mating type locus, or (iv) they can sexually reproduce but carry only one *MAT* locus (34). The Ac*MAT1-1* locus appears not to have a *MAT1-2-1* homologue, which can be found in *MAT* loci of homothallic members, either fused or located in close proximity to the α-box domain gene in Soradiomycetes and Dothidiomycetes (15, 52, 55). Thus, homothallism due to a fused *MAT* locus can be ruled out. In the genomes of the homothallic *Emericella nidulans* and

Neosartorya fischeri, *MAT1-1* and *MAT1-2* loci are on separate chromosomes, and in the homothallic *Chaetomium globosum*, in addition to a *MAT1-1* locus, a *MAT1-2* locus on another supercontig was identified previously (15, 21, 61). Unfortunately, the genome sequence of *A. chrysogenum* is still not yet available, and therefore, the possibility of an unlinked second *MAT* locus encoding a MAT1-2-1 homologue in the *A. chrysogenum* genome cannot be excluded. It may also be possible that *A. chrysogenum* has silent *MAT* loci and can undergo mating type switching, as described previously for several filamentous ascomycetes (14, 34). Interestingly, in some homothallic species of the genus *Neurospora* (*Neurospora africana*, *Neurospora lineolata*, *Neurospora galapagonensis*, and *Neurospora dodgei*), only the *MAT1-1* locus is present in the genome (23, 24, 53). It has been speculated that in these species, either sexual reproduction does not require the HMG domain protein encoded by the *MAT1-2* locus or the function of this protein can be replaced by the *MAT1-1-3*-encoded HMG protein (40). Alternatively, *A. chrysogenum* may be a heterothallic fungus with a predominantly homothallic life cycle. Unmated MAT1-1 strains of the heterothallic ascomycete *Sordaria brevicollis* can form ascospores, whereas MAT1-2 strains are able to form only barren fruiting bodies (59). Thus, the unequal distribution of mating types in *A. chrysogenum* may be due to a preference for the unisexual mating of MAT1-1 strains. More than 99% of clinical and environmental isolates of the heterothallic basidiomycete *Cryptococcus neoformans* are MATα, and it was shown previously that predominantly haploid MATα strains can produce basidiospores by unisexual mating, a modified form of sexual reproduction that involves diploidization and meiosis (36, 37). Quantitative-trait-locus mapping revealed that the MATα allele enhances hyphal growth during unisexual mating as a quantitative trait locus and may explain why MATα isolates predominate in nature (35).

Sequence analysis of all Ac*MAT1-1* genes provided no evidence of loss-of-function mutations, indicating that the *MAT* locus may still contain transcriptionally expressed functional genes that have not been subjected to pseudogene formation, which would be expected to occur if *A. chrysogenum* had evolved to be asexual. In addition, we showed that *A. chrysogenum* possesses and transcriptionally expresses some genes crucial for pheromone processing and signal transduction. This finding further supports our hypothesis of a sexual cycle in *A. chrysogenum*. Homologues of the *S. cerevisiae FAR1*, *STE5*, *KSS1*, *DIG1*, and *DIG2* genes were not identified in the *A. chrysogenum* EST library. However, since these genes were also absent in *Penicillium marneffei*, *Emericella nidulans*, and *Aspergillus fumigatus* (76), filamentous ascomycetes may use alternatives to the proteins encoded by these genes in pheromone signal processing and transduction.

The *MAT1-1* locus of *A. chrysogenum* induces perithecial and ascospore development in the heterothallic fungus *P. anserina*. A functional characterization of the Ac*MAT* genes was carried out by heterologous expression in a PaΔMAT strain. Interestingly, PaΔMAT-Ac*MAT1-1*^{ect} strain fungi carrying an ectopic copy of the Ac*MAT1-1* locus developed fertile perithecia containing rosettes of asci with ascospores (Fig. 6). These results indicate that the Ac*MAT1-1* locus is not only structurally but also functionally similar to the Pa*MAT1-1* (*MAT*$^{-}$) locus and are consistent with previous findings showing that cloned *MAT* genes of homothallic and asexual species are functional in heterothallic species (3, 24, 55, 56, 67). Like the asci and ascopores produced by the transgenic strains in this study, asci and ascospores produced by the transgenic PaΔMAT and *Cochliobolus heterostrophus* ΔMAT strains expressing heterologous *MAT* genes were found previously to be less abundant than those produced by wild-type strains (3, 51). The AcMAT1-1-1 and AcMAT1-1-3 proteins have highly conserved α-box domain and HMG box DNA-binding motifs (Fig. 2) and therefore may bind to the same target genes driving fertilization and postfertilization processes. However, interaction with other transcription factors, e.g., MCM1 and STE12, involved in fruiting body development and ascosporogenesis in Sordariales (43, 44) may be affected, thereby altering the transcriptional expression of the target genes and leading to decreased ascus and ascospore production. In our study, RT-PCR analysis revealed that ectopic integrated Ac*MAT1-1* genes were transcriptionally expressed in *P. anserina* (Fig. 5C). In contrast, in a previous study, the *Aspergillus fumigatus MAT1-2-1* mating type gene ectopically introduced into an *Emericella nidulans* ΔMAT strain was not properly expressed and consequently did not support either fruiting body or ascospore development. The *Aspergillus fumigatus MAT1-2-1* gene locus is able to confer full fertility only when it is driven by the *Emericella nidulans MAT1-2-1* promoter and integrated by homologous recombination into the *Emericella nidulans MAT* locus (56).

Interestingly, an analysis of the ascospore progeny produced by the transgenic PaΔMAT strain showed that only 2 of 30 randomly isolated spores were biparental strains expressing the *MAT* genes from both loci. All other spore isolates were uniparental strains expressing only the Ac*MAT1-1* genes (Fig. 5). The production of uniparental progeny by *P. anserina* can often be observed among strains with a mutated *MAT1-1-3* (*SMR2*) gene. In this situation, the genetic markers of the uniparental progeny come exclusively from the parent containing the mutation. Viable ascospores are formed after a haploid meiosis event during which the random segregation of chromosomes results by chance in even distribution (70). One of the mutations leading to uniparental progeny is *smr2*(E202A) (4, 70). The analysis of an amino acid alignment of the AcMAT1-1-3 and PaMAT1-1-3 (SMR2) proteins revealed that the amino acid residue E202 in PaMAT1-1-3 corresponds to A148 in AcMAT1-1-3. In *P. anserina*, the wild-type MAT1-1-3 (SMR2) protein seems to repress MAT1-2 (MAT^{+}) fertilization functions in MAT1-1 (MAT^{-}) sexual organs by interaction with MAT1-1-1 (FMR1). According to the internuclear recognition model (70), nuclei carrying a mutated *MAT1-1-3* (*SMR2*) gene express both the *MAT1-1* and *MAT1-2* functions required for internuclear recognition and trigger self-recognition.

Together, the identification of conserved components of the pheromone response pathway and the functional conservation of the *MAT* genes provide the first evidence to suggest a sexual cycle in *A. chrysogenum*. To date, there are no reports on possible reasons for the presence of functional *MAT* genes in supposedly asexual filamentous fungi. However, one explanation is that some of the supposedly asexual species may have a cryptic sexual cycle (8). Another possibility is that sexual reproduction is a rare event that is hard to detect (74) and thus remains to be described. Nevertheless, the activation of a sex-

ual cycle in *A. chrysogenum* would increase options for alternative strain improvement strategies.

Further studies are needed to search the *A. chrysogenum* genome for other sequences known to be necessary for meiotic recombination in other fungi and then to examine their function. To clarify whether the reproductive mode of *A. chrysogenum* is heterothallic or homothallic, the *A. chrysogenum* genome should also be further analyzed for the presence of a *MAT1-2-1* mating type gene homologue. Since all *A. chrysogenum* strains analyzed in this study were derived from type culture collections, we cannot exclude the possibility that they may have lost fertility during the long time of storage. Thus, future studies will examine *A. chrysogenum* strains isolated directly from the environment for past or ongoing meiotic recombination. Finally, whether mating type proteins in *A. chrysogenum* regulate other genes not directly involved in sexual reproduction remains to be elucidated.

ACKNOWLEDGMENTS

We thank Ingeborg Godehardt, Kerstin Kalkreuter, Stefanie Mertens, and Susanne Schlewinski for excellent technical assistance and Hubert Kürnsteiner, Ernst Friedlin, Thomas Specht, and Ivo Zadra (Kundl, Austria) for the *A. chrysogenum* EST sequences and for fruitful discussion. We are grateful to Evelyne Coppin (Institut de Genetique et Microbiologie, CNRS-Université Paris Sud, Orsay, France) for providing the PaΔMAT strain.

We acknowledge financial support from Sandoz GmbH (Kundl, Austria) and from the Christian Doppler Society (Vienna, Austria).

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