Mating-Type Genes From the Homothallic Fungus *Sordaria macrospora* Are Functionally Expressed in a Heterothallic Ascomycete

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ABSTRACT

Homokaryons from the homothallic ascomycte *Sordaria macrospora* are able to enter the sexual pathway and to form fertile fruiting bodies. To analyze the molecular basis of homothallism and to elucidate the role of mating-products during fruiting body development, we cloned and sequenced the entire *S. macrospora* mating-type locus. Comparison of the Sordaria mating-type locus with mating-type idiomorphs from the heterothallic ascomycetes *Neurospora crassa* and *Podospora anserina* revealed that sequences from both idiomorphs (*A*/*a* and *mat*−/*mat*+, respectively) are contiguous in *S. macrospora*. DNA sequencing of the *S. macrospora* mating-type region allowed the identification of four open reading frames (ORFs), which were termed *Smt-a1*, *SmtA-1*, *SmtA-2* and *SmtA-3*. While *Smt-a1*, *SmtA-1*, and *SmtA-2* show strong sequence similarities with the corresponding *N. crassa* mating-type ORFs, *SmtA-3* has a chimeric character. It comprises sequences that are similar to the *A* and *a* mating-type idiomorph from *N. crassa*. To determine functionality of the *S. macrospora* mating-type genes, we show that all ORFs are transcriptionally expressed. Furthermore, we transformed the *S. macrospora* mating-type genes into *mat*− and *mat*+ strains of the closely related heterothallic fungus *P. anserina*. The transformation experiments show that mating-type genes from *S. macrospora* induce fruiting body formation in *P. anserina*.

THE regulation of sexual reproduction is one of the central processes in the life cycle of most eukaryotes. The recent cloning and characterization of sequences specifying mating types from mycelial fungi has allowed insights into the regulatory processes controlling sexual reproduction in ascomycetes (reviewed in GLASS and NELSON 1994; METZENBERG and RANDALL 1995; NELSON 1996). Mating behavior in ascomycetes can be either homothallic or heterothallic. In contrast to the well-studied mating types in heterothallic filamentous ascomycetes, the genetic basis of homothallism remains largely uncharacterized. No instance of mating-type switching or interconversion that occurs in homothallic yeasts have been reported for Neurospora (PERKINS 1987).

The homothallic ascomycete *Sordaria macrospora* (Pyrenomycetidae, Sordariaceae) has been an excellent model system for studying not only meiotic pairing and recombination (ZICKLER 1977) but also fruiting body development (ESSER and STRAUB 1958; HESLOT 1958). In contrast to heterothallic species of the Sordariaceae, in *S. macrospora*, a homokaryotic ascospore gives rise to hyphae that can enter the sexual reproductive pathway and produce fruiting bodies (perithecia) enclosing the ascospore progeny.

In heterothallic Sordariaceae, such as *Neurospora crassa* and *Podospora anserina*, a homokaryotic ascospore is not capable of completing the sexual cycle. *N. crassa* and *P. anserina* are composed of two mating-type populations, designated *A* and *a* or *mat*− and *mat*+, respectively. Mating occurs only between sexual structures of opposite mating type. The sequences conferring mating behavior in *N. crassa* and *P. anserina* consist of dissimilar DNA sequences (idiomorphs), which are present at a homologous locus in the mating partners (GLASS *et al.* 1988; PICARD *et al.* 1991). Database searches revealed that conserved DNA binding motifs, such as HMG boxes, α domains, or amphipathic alpha helical domains, are found in all of the mating-type products (GLASS *et al.* 1990a; STABEN and YANOFSKY 1990; DEBUCHY *et al.* 1993; FERREIRA *et al.* 1996). For this reason, the mating-type genes are believed to encode master transcriptional regulators that control sexual development in filamentous ascomycetes (NELSON 1996).

In an effort to elucidate the phenomenon of homothallism in mycelial ascomycetes, homothallic members of Sordariaceae were probed with the *A* and *a* mating-type sequences from *N. crassa*. The analysis revealed that sequences hybridizing to the *N. crassa* mating-type locus are conserved in both heterothallic and homothallic Sordariaceae species. Two groups of homothallic species could be distinguished in this study: the first group contains only sequences similar to the *N. crassa* *A* idiomorph (*A* type) and a second group contains sequences similar to both the *A* and the *a* idiomorphs (*A*/*a* type) (GLASS *et al.* 1988, 1990b).

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Recently the mating-type locus from *N. africana*, which is a representative of *A*-type homothallic Sordariaceae, has been examined in some detail and the *N. crassa mt A-1* homologue has been sequenced and was shown to function as mating activator in *N. crassa* (GLASS and SMITH 1994). In addition, the mating-type loci of selected members of the *A*/*a*-type Sordariaceae have been compared with the mating-type idiomorphs of *N. crassa* by means of Southern hybridization. However, a direct linkage of the *A*- and *a*-specific sequences could not be confirmed in this study (BEATTY *et al.* 1994).

To our knowledge, this paper provides the first molecular description of the entire mating-type locus from a homothallic mycelial fungus. The ascomycete *S. macrospora* is a member of the *A*/*a*-type homothallic Sordariaceae, and, due to the development of optimized molecular tools, this fungus became more suitable for molecular genetic investigations (WALZ and KÜCK 1995; PÖGGELER *et al.* 1997). We show that the four mating-type genes in *S. macrospora* are transcriptionally expressed and demonstrate that the introduction of homothallic mating-type genes from *S. macrospora* leads to the initiation of fruiting body development in the heterothallic fungus *P. anserina*. The result indicates that both *A*- and *a*-specific mating-type genes are functionally expressed in homothallic species. Our findings provide new aspects to the understanding of the molecular basis of homothallism and the involvement of mating-type genes in fruiting body development in ascomycetes.

MATERIALS AND METHODS

Bacterial and fungal strains: *S. macrospora* strain k-hell (3346) from our laboratory collection has a wild-type phenotype (ESSER and STRAUB 1958). Genomic DNA from strain 3346 was used for construction of the indexed *S. macrospora* cosmid library (PÖGGELER *et al.* 1997). The *P. anserina* strains *mat*– (700005) and *mat*+ (700004) were derived from homokaryotic spores of *P. anserina* wild-type strain s (ESSER 1974) containing only one of the two mating types and were used as hosts for transformation experiments. Standard media and conditions for the cultivation of *P. anserina* were as described by ESSER (1974) and OSIEWACZ *et al.* (1991). *Escherichia coli* strain XL1-Blue MR (Stratagene, Heidelberg, Germany) served as a recipient strain for recombinant cosmids, and strain XL-1 Blue (BULLOCK *et al.* 1987) was used for propagation of recombinant plasmids.

Cloning of the *S. macrospora* mating-type locus: An indexed genomic cosmid library from *S. macrospora* was screened by a rapid method (PÖGGELER *et al.* 1997) with heterologous probes carrying sequences from the *N. crassa A* or *a* idiomorph probes. For this purpose, a 0.6-kb *Nco*I/*Eco*RI fragment from plasmid pmtAG-2, which carries a 1.2-kb *Eco*RI/*Bcl*I fragment from the *A* mating-type specific region (GLASS *et al.* 1990a), was used. Similarly, a 1.1-kb *Pst*I/*Nco*I fragment from plasmid pCSN4, which contains a 1.9-kb *Bam*HI/*Eco*RV fragment from then *a* mating-type specific region (STABEN and YANOWFSKY 1990), served as *a* mating-type specific probe. Two out of 96 cosmid pools of 48 wells each hybridized to the *A*-specific as well as to the *a*-specific probe from *N. crassa*. A colony filter hybridization between clones from the two pools was subsequently hybridized with the *A*- and *a*-specific probes giving rise to three labeled clones designated D1, D9 and H2. Cosmid DNA from these three clones was prepared according to the protocol provided by Stratagene together with cosmid sCos.

Standard methods of molecular biology were used according to SAMBROOK *et al.* (1989). Subcloning of hybridizing DNA fragments from cosmid clone D1 resulted in the construction of pBluescript (KS+) (Stratagene) derivatives (Figure 1 and Table 1).

Oligonucleotides: Oligonucleotides (Table 2) were synthesized for use as sequencing primers and/or primers for specific reverse transcription PCR (RT-PCR) amplifications.

DNA sequencing and sequence analysis: Double-stranded sequence reactions were performed with a sequenase kit (Amersham/USB Cleveland) under conditions recommended by the manufacturer. An ordered set of deletion clones was prepared using a nested deletion kit (Pharmacia, Freiburg, Germany). Clones were sequenced on both strands using the universal or reverse sequencing primer. Individual sequencing primers (Table 2) were synthesized in regions where clone

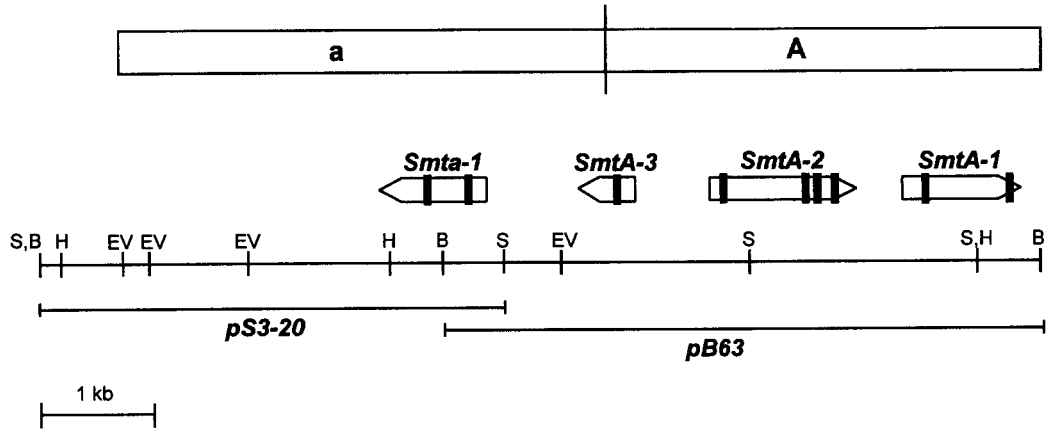

FIGURE 1.—Physical map of the *S. macrospora* mating-type locus. The line showing restriction enzyme sites covers the 8810-bp region that was sequenced in this investigation. ⇒, position and orientation of the ORFs; ■, intronic sequences. *A*- and *a*-specific regions are indicated. Lines below the map represent restriction fragments that were subcloned and sequenced or used for transformation of *mat*– and *mat*+ strains from *P. anserina* (see also Table 1).

TABLE 1

Description of plasmids used in this investigation

Plasmid	Insert/description	Vector
pB63	5.2-kb *Bam*HI fragment of cosmid *D1*	pBluescript/KS$^+$
pS3-20	4.1-kb *Sal*I fragment of cosmid *D1*	pBluescript/KS$^+$
p50-4	RT-PCR product with primers nos. 814 and 778 (*Smta-1*)	pBluescript/KS$^+$
p52-16	RT-PCR product with primers nos. 831 and 832 (*SmtA-2*)	pBluescript/KS$^+$
p54-35	RT-PCR product with primers nos. 750 and 785 (*SmtA-1*)	pBluescript/KS$^+$
p56-34	RT-PCR product with primers nos. 829 and 883 (*SmtA-3*)	pBluescript/KS$^+$
pB122	5.2-kb *Bam*HI fragment of cosmid D1	pANsCos 1
pH8-302	5.2-kb *Hin*dIII fragment of cosmid D1	pBChygro
p26-36	4.2-kb *Eco*RV-*Bam*HI fragment of cosmid D1	pBChygro
p37-74	4.2-kb *Eco*RV-*Bam*HI fragment of cosmid D1; deletion of 41 bp (positions 8268–8308) in the *SmtA-1* ORF	pBChygro
p9-11	2.8-kb *Eco*RV fragment of cosmid D1	pBChygro
p33-6	2.8-kb *Eco*RV fragment of cosmid D1; integration of 18 bp (oligo No. 783) in the *Smta-1* ORF at position 3615	pBChygro
p51-4	2.8-kb *Eco*RV fragment of cosmid D1; deletion of 32 bp (positions 3600–3631) in the *Smta-1* ORF	pNP2-1

coverage was insufficient. DNA sequence was analyzed with the Husar/Genius computer program software package (EMBL, Heidelberg) and will appear in the EMBL database under accession No. Y10616.

Complementation transformation of *P. anserina*: Preparation and transformation of *P. anserina* protoplasts was performed as described previously (OSIEWACZ *et al.* 1991). Recombinant plasmids used for complementation transformation of *P. anserina* were derivatives of plasmid pBChygro (SILAR 1995), plasmid pNP2–1 (OSIEWACZ and NUBER 1996) and plasmid pANsCos1 (OSIEWACZ 1994) (Table 1).

Genomic DNA preparation and analysis: Genomic DNA from *P. anserina* was prepared according to a method described previously (HERMANNS *et al.* 1995). DNA hybridizations were carried out under stringent conditions using the DIG labeling and detection system from Boehringer Mannheim (Germany) according to the manufacturer's recommendation.

cDNA analysis: Total RNA was isolated from 10 g (fresh weight) of mycelia grown for 3 days in CCM-medium (WALZ and KÜCK 1995). The mycelia was ground with liquid nitrogen in a mortar, and the powder was mixed with an equal amount of boiling extraction buffer (KEMPKEN and KÜCK 1996). The supernatant was extracted once with phenol and twice with phenol-chloroform-isoamyl alcohol (25:24:1). After an additional chloroform-isoamyl alcohol (24:1) extraction, nucleic acids were ethanol precipitated and the pellet was dissolved in distilled H_2O. RNA was selectively precipitated by adding LiCl to reach a final concentration of 2 M. The Poly Atract mRNA isolation system (Promega, Heidelberg, Germany) was used to enrich poly(A)$^+$ RNA. cDNAs were synthesized from 1.0 μg of poly(A)$^+$ RNA using the reverse transcriptase-PCR (RT-PCR) protocol described by KEMPKEN and KÜCK (1996). PCR was performed in accordance with the method of SAIKI *et al.* (1988) using the primers Nos. 778 and 814 for the amplification of *Smta-1* cDNA, primers Nos. 829 and 883 for *SmtA-3* cDNA, primers Nos. 831 and 832 for *SmtA-2* cDNA, and primers Nos. 750 and 785 for *SmtA-1* cDNA. The amplified products were separated from unincorporated nucleotides and primers by using a PCR purification kit (Boehringer) and subsequently cloned into vector pBluescript/KS$^+$ linearized with *SmaI* (Table 1). The cDNAs were sequenced with specific primers deduced from the genomic DNA sequence.

Microscopic investigations: Morphologies of protoperithecia and perithecia from *P. anserina* wild-type, recipient, and transformant strains, were examined after treatment of cultures grown for 14 days on BMM agar medium with 10 ml fixative (ethanol, acidic acid 3:1 by volume) for 10 hr. Subsequently, plates were washed twice with 70% ethanol, and pieces of mycelium were transferred to buffer on a slide. Pictures were taken with Kodak Technical Pan film through an Zeiss Axiophot microscope at ×200. Quantitative micro-

TABLE 2

Numbers and nucleotide sequences of oligonucleotides used in this investigation

Oligo no.	Sequence 5′→3′	Position (nucleotides) in the *S. macrospora mat*-locus
750	ATGTCCAGCGTCGATCAAATCGTC	7633–7656
778	TCACAGTGGAAATGAGCGCTGGGT	2718–2741
783	GATCCCCGAATTCGGG	Linker
785	CTATGCTCCTCCATTGAATC	8672–8653
814	ATGGAAGACAACTTGATGCACC	3971–3951
829	ATGTCTGCCCCAGACGTTAG	5188–5168
831	ATGGAGCTCATCAACACGCAA	5917–5937
832	TCAAATGTGATAAACGTGCTCCTC	7226–7203
883	CGTCCTATTTTAGAGATTGC	4661–4680

scopic morphometry was done at the same magnification by measuring the diameter of 250 protoperithecia and perithecia from, each, wild-type, recipient, and transformed strains of *P. anserina* with an ocular micrometer.

RESULTS

Cloning of the *S. macrospora* mating-type locus: Hybridization analysis showed that the homothallic ascomycete *S. macrospora* contains sequences with similarity to both the *N. crassa A* and *a* idiomorph (GLASS *et al.* 1990b). Using a rapid screening procedure (PÖGGELER *et al.* 1997), the *S. macrospora* mating-type region was isolated by hybridizing the pooled cosmid DNA from an indexed genomic cosmid library with heterologous probes specific for the *N. crassa A* and *a* mating-type idiomorphs. This procedure allowed the isolation of three cosmids: D1, D9, and H2. An internal 19-kb *Eco*RI fragment contained in all of the above mentioned cosmids hybridized with the *N. crassa A*-specific as well as with the *a*-specific probe. The fragment pattern and insert size of D9 and H2 were identical. Cosmids D1 and D9/H2 contain an insert of 37.7 and 33.4 kb, respectively. Comparative Southern blot analysis of the *S. macrospora* genomic DNA and cosmid DNAs revealed that the cloned DNAs are not rearranged with respect to their true genomic organization and that the *S. macrospora* mating-type genes all have a single copy in the genome (data not shown). The deduced physical map of the genome in the mating-type region is shown in Figure 1.

DNA sequence analysis: To analyze the structural organization of the mating-type region, we first constructed several plasmids carrying specific restriction-fragments from this region (Figure 1). The complete nucleotide sequence of the *Bam*HI (plasmid pB63) and of the *Sal*I (plasmid pS3-20) fragments, which together cover 8815 bp of the mating-type region, was determined on both strands. Computer analysis of this sequence combined with cDNA isolation and sequencing resulted in the identification of four ORFs which, in accordance with the mating-type genes from *N. crassa*, were termed *Smta-1*, *SmtA-3*, *SmtA-2* and *SmtA-1*. The *Smta-1* ORF encodes a polypeptide of 288 amino acid residues and contains two introns of 53 and 57 bp, which exhibit typical *S. macrospora* consensus splice sites (PÖGGELER 1997). A search of the EMBL databases with the TFASTA program (PEARSON and LIPMAN 1988) showed significant similarities between the *Smta-1* ORF and the *N. crassa mta-1* ORF (STABEN and YANOFSKY 1990) or the *FPR1* ORF of *P. anserina* (DEBUCHY and COPPIN 1992). The *Smta-1* ORF derived amino acid sequence shows 82.3 and 19.1% identity with the corresponding sequences from *N. crassa* and *P. anserina* (Figure 2A). A DNA binding motif found in the translation products of *Smta-1* was also detected in the translation products of *mta-1* and *FPR1* (Figure 2A). It shows a region of similarity to known DNA binding sequences found within the *Cochliobolus heterostrophus* MAT-2 (TURGEON *et al.* 1993), *Schizosaccharomyces pombe* MAT Mc (KELLEY *et al.* 1988), and human sex-determining factor SRY as well as in the closely related human SOX proteins (SINCLAIR *et al.* 1990; DENNY *et al.* 1992). All of these proteins are members of the HMG (high mobility group) family of DNA binding proteins (reviewed in GROSSCHEDL *et al.* 1994).

The coding sequence proposed for the mating-type gene *SmtA-3* (Figure 3A) initiates at position 5188 and terminates at position 4788. DNA sequence comparison shows that the 5′ part of this gene has high homology with the *N. crassa A* idiomorph. This similarity is markedly reduced further downstream from position 4860 of the *S. macrospora* sequence. The 3′ sequence of *SmtA-3* reveals a significant homology with the *N. crassa a* idiomorph (Figure 3B). *SmtA-3* is interrupted by one intron of 54 bp and encodes a polypeptide of 116 amino acids. Comparison of the SMTA-3 polypeptide with proteins from the database shows a high degree of identity of 89.0% to the 91 N-terminal amino acids of the corresponding mating-type protein MTA-3 of *N. crassa*. The *N. crassa* MTA-3 and the *P. anserina* SMR2 proteins both contain a HMG domain (DEBUCHY *et al.* 1993; FERREIRA *et al.* 1996). This domain is lacking in the *S. macrospora* SMTA-3 protein (Figure 2B).

The *SmtA-2* ORF encodes a polypeptide of 360 amino acids. The ORF is interrupted by four introns of 57, 71, 47 and 55 bp, which display typical *S. macrospora* consensus splice sites (PÖGGELER 1997). The deduced *SmtA-2* amino acid sequence was compared with databases and was shown to be 71.3 and 12.5% identical to the *N. crassa mtA-2* ORF and the *P. anserina SMR1* ORF, respectively (Figure 2C). No other sequences with significant similarity to the *SmtA-2* ORF were found.

The coding sequence proposed for the fourth gene, *SmtA-1*, can be translated into a protein of 307 amino acid residues. Two introns of 59 and 60 bp showing typical *S. macrospora* splice sites were identified. Comparison of the predicted amino acid sequence with known sequences from databases revealed that the *SmtA-1* gene is 72.3 and 30.6% identical to that of the *N. crassa mt A-1* gene or to that of the *FMR1* ORF from *P. anserina* (Figure 2D). The DNA binding motif found in the SMTA-1 polypeptide (Figure 2D) also shows similarities to a known DNA binding sequence in the *Saccharomyces cerevisiae* matα1 protein (ASTELL *et al.* 1981; TATCHELL *et al.* 1981).

RT-PCR analysis of the *S. macrospora* mating-type genes: To obtain direct evidence for the transcription of the mating-type genes in *S. macrospora*, we performed mRNA analysis of the four genes. Total RNA was isolated from liquid cultures of *S. macrospora* grown at 27° for 3 days after inoculation. In Northern (RNA) hybridizations, using enriched poly(A) mRNA, only weak signals could be detected. Therefore we decided to employ the more sensitive RT-PCR technique. Enriched

poly(A) mRNA was treated with RNase-free DNase to remove any DNA contamination. Using reverse transcriptase and random hexamer primers, cDNAs were constructed. PCRs were performed with specific primers (Table 2) from cDNAs, and DNA fragments were amplified as a control. In all cases, the RT-PCR product obtained from the cDNA template had a slightly increased electrophoretic mobility compared with that of the PCR product derived from a DNA control (data not shown). This result could be explained by the presence of intronic sequences in the DNA. The cDNAs from the RT-PCR experiments were cloned (Table 1), sequenced and compared with the genomic DNA sequence. The sequence analysis of the cDNA from *Smta-1*, *SmtA-3*, *SmtA-2* and *SmtA-1* revealed that all intron sequences in the genomic DNA that were predicted from the comparison with the *N. crassa* mating-type genes are lacking in the cDNAs. Therefore, the existence of a spliced mRNA from all *S. macrospora* mating-type genes indicates that all mating-type genes identified in the mating-type locus of *S. macrospora* are transcriptionally active.

Expression of derivatives of the *S. macrospora* mating-type locus in *P. anserina*: To further investigate the functional conservation of the *S. macrospora* mating-type genes, we transformed the cosmid clones D1 and D9, both containing the entire mating-type locus from *S. macrospora*, into *P. anserina mat*− and *mat*+ strains. After introduction of the *S. macrospora* mating-type information by DNA-mediated transformation, 60% of the *mat*− transformants and 37% of the *mat*+ transformants were capable of inducing fruiting body development without crossing to a mating partner with the opposite mating-type. Unfertilized protoperithecia of *mat*− and *mat*+ recipient strains are small in comparison with fertile heterokaryotic perithecia produced in the *mat*− × *mat*+ cross (Table 3). After transformation with cosmid D1, both *mat*− and *mat*+ strains became capable of producing perithecia (Table 3). Like the fertilized wild-type strain, transformants develop large, flask-shaped perithecia from ball-shaped protoperithecia. However, the transformant fruiting bodies do not form tufts of hairs near the ostiole, which is a characteristic feature of perithecia from *P. anserina*. The most striking difference between the fruiting bodies from transformant strains and wild-type strains is that spore formation was only observed in the heterokaryotic wild-type perithecia derived from crosses. In contrast, the homokaryotic perithecia from the transformants contain a gelatinous mass showing no structures such as croziers, asci, or spores. Size differences between protoperithecia and perithecia produced from wild-type crosses, recipient strains, and transgenic strains were assayed by measuring the diameter of 250 fruiting bodies with an ocular micrometer (Table 3). In *mat*− and *mat*+ recipient strains, hardly any protoperithecia with a diameter of >120 μm were detected (Table 3). A large number of fertile perithecia are produced in the *mat*− × *mat*+ cross where the mean diameter is 91.3 μm, and 24.4% of the perithecia are >120 μm (Table 3). The transformants Ts^+D1-1.13 and Ts^-D1-3.20, which became capable of generating perithecia, exhibit a similar frequency of large fruiting bodies (>120 μm) 26.4 and 27.6%, respectively (Table 3). Nevertheless, all perithecia produced by the transformants were sterile and contained neither spores nor asci.

To investigate the function of single *S. macrospora* mating-type genes in more detail, DNA-mediated transformations were performed with *P. anserina mat*− and *mat*+ strains to separately introduce the *A*- and *a*-specific *S. macrospora* mating-type genes. The introduction of *SmtA-1* (pB122, p26-36; Table 1) into the *mat*+ strain gave rise to transformants that produced sterile perithecia (Ts^+pB122-1.7, Ts^+pB122-1.9, Ts^+p26-36-1.17; Table 3). The mean diameter of the perithecia from these three transformants is similar to those in the wild-type cross. This is further confirmed by the percentage of large perithecia (>120 μm), which range from 12 to 32.8% in these transformants. Similarly, perithecia with the same characteristics were developed by the *mat*− transformants Ts^-p9-11-1.13, Ts^-p9-11-1.23 after the transformation with plasmid p9-11 containing the entire *Smta-1* gene (Table 3). In contrast, transformants Ts^-p9-11-1.9 and p9-11-1.5, which should carry the same plasmid, show no perithecial development. The mean diameter of the protoperithecia is only 51.5 and 54.7 μm, respectively. In addition, only 0.8 or 1.2%, respectively, of the fruiting bodies are >120 μm in these transformants. A similar order of magnitude of large protoperithecia appears in the recipient *mat*− strain. Fruiting bodies could be detected in neither *mat*− strains nor in *mat*+ strains after the transformation of plasmid pH8-302, encoding only the SMTA-2 protein and SMTA-3 protein.

Molecular analysis of selected *P. anserina* transformants: To test a possible correlation between the number and genomic arrangement of integrated plasmid copies and the ability of the transformants to produce fruiting bodies, we isolated total DNA from hygromycin B resistant *P. anserina mat-* transformants generated by introduction of plasmid p9-11. Two of the selected transformants are able to produce a large number of perithecia (Ts^-9-11-1.13, Ts^-9-11-1.25), one transformant produces only a few perithecia (Ts^-9-11-1.24), and two strains are unable to generate fruiting bodies at all (Ts^-9-11-1.3, Ts^-9-11-1.5). In a first set of Southern hybridization experiments, we used the complete transforming plasmid p9-11 as a probe. As expected, this probe detected sequences in the genomic DNA of all five transformants. Moreover, the digestion of DNA from the two transformants Ts^-9-11-1.13, Ts^-9-11-1.25 with *Cla*I, an enzyme with a single recognition site in the transforming vector, led to a single DNA fragment hybridizing with a higher intensity (marked by an asterisk) than the other fragments (Figure 4).

A

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            10        20        30        40        50        60
             .         .         .         .         .         .
  1                                                    MEDNLMH SMTa-1
  1                                                    MDGNSTH MT a-1
  1 MAAFNFEAFSLTPQGSTISAAPRPAAPAIDRTVQQQCGSFGYGNRAFQFDFASLESLPED FPR1
                                                       .. .  .

            .         .         .         .         .         .
  8 PAPTSAELRATMAWSRISNQLGHWNDRKIIAIPLSDFTIAHPDIHAGIVAEYKKATGEEG SMTa-1
  8 PAP---NLKTTMAWSRISNQLGHWNDRKVIAIPLSDFLNTHPDIQSGIIAEFKKATGEEG MT a-1
 61 ANPGLTEVLTAKYWNHFSIQLGHWNTLKVIVLDAQMFSIM-PDHTKKGVLNTMNTTV--- FPR1
    ..*   .. ....*...*.******..***..... . .**.  ..... ...*....

            .         .         .         .         .         .
 68 MFARDTEALEIMLLGPVKLFKPDSVVVEGNLFWDPKGIHAEAPKQQQKKKA***IPRPPNAY SMTa-1
 65 MFARDPESLGIMLLGPVKLFKPDSVVVDGNLFWDPKGIHASAPKEQQKK-A***IPRPPNAY MT a-1
117 ---------------------------------WDPKKRHVQATAE-----A***IPRPPNAY FPR1
    ..... . . .....................****..*. *....... **********

            .         .         .         .         .         .
128 ***ILYRKDHHRQIREQNPGLHNNEISVIVGNMWRDEQPHIRDKYFSMANEIKARLLLDNPDY*** SMTa-1
124 ***ILYRKDHHREIREQNPGLHNNEISVIVGNMWRDEQPHIREKYFNMSNEIKTRLLLENPDY*** MT a-1
140 ***ILYRKDQQAALKAANPGIPNNDISVMTGGMWKKESPEVRAEYQRRASEIKAKLMSAHPHY*** FPR1
    ******... ....****.**.***..*.**..*.*..* .*. ...***..*.. .*.*

            .         .         .         .         .         .
188 ***RYNPRRSQDIRR***RVSPYLKIKLLNY-----DGNGNLLWG----TVNAEDAALIRTHFHGV SMTa-1
184 ***RYNPRRSQDIRR***RVSPYLKIKLLNY-----DVNGNLLWG----TVNAEDAALIRTHFHGV MT a-1
200 ***RYVPRRSSEIRR***RAPRRNRAQEVANASPIGENSGAPIVGNPIVTTMEQQQPLPDISIAPN FPR1
    **.****..****.............     . .*....*    *.......*........

            .         .         .         .         .         .
239 VRVEETDEGCRIVCRPVAGSRKLRAANVDTWMPRYTVDATPISEEDEAA*            SMTa-1
235 VRVEEMDDGCRIVCRPVAGSRKLRAAVVDTWMPRYTVDTTPVTEDDDAQAFNFNDPLGG- MT a-1
240 QEITKDNDVSHLI-----DPPHVFSGQITELMPDVANFLPPMIREGWSPLHDFRAVLNGH FPR1
    ..... ..................... ....**..... .*  ... .    .   . .

            .         .         .         .         .         .
    ------------------------------------------------------------ SMTa-1
294 -AYFPLNEHLWITVNQNPPFNAPPPNPNP-HLDFVHPDGMEAVVHNVQNMIAQVQEANEA MT a-1
295 TGNNGVDCALTPESESQDDFVGTPSSTMPDNSAFDWITGTE---EDLAQIFGQF*----- FPR1
                    .    .   .  .          .

            .         .         .         .         .         .
    ------------------------------                           SMTa-1
352 AALTLPPPPPLRLLSLRLWLMIPLTQLSFPL*                          MT a-1
    ------------------------------                           FPR1
```

FIGURE 2.—Comparison of the deduced polypeptides of the mating-type genes from *S. macrospora, N. crassa,* and *P. anserina.* (A) Alignment of the deduced amino acid sequence of the *Smta-1* ORF from *S. macrospora* with the mating-type polypeptides MTa-1 from *N. crassa* and FPR1 from *P. anserina.* The HMG DNA binding region is indicated in bold italics. (B) Deduced amino acid sequence from the *S. macrospora SmtA-3* gene compared with the *N. crassa* MT A-3 protein and the *P. anserina* SMR2 protein. The HMG DNA binding region found in MTA-3 and SMR2 is indicated in bold italics. (C) Comparison of the deduced amino acid sequence from the *S. macrospora SmtA-2* gene with the *N. crassa* MT A-2 protein and the *P. anserina* SMR1 protein. (D) Deduced amino acid sequence of the *S. macrospora SmtA-1* gene compared with the MT A-1 protein from *N. crassa* and the FMR1 protein from *P. anserina.* The α domain DNA binding region is indicated in bold italics Alignments were made using the MULTALIGN program provided by the HUSAR/genius computer program software package. *N. crassa* sequences data are taken from GLASS *et al.* (1990a) and FERREIRA et al. (1996); *P. anserina* sequences data are taken from DEBUCHY and COPPIN (1992) and DEBUCHY *et al.* 1993. Dashes indicate gaps introduced for optimal alignment. The numbers at the left refer to the amino acid position in the corresponding protein. The consensus line at the bottom indicates conserved amino acid residues in the aligned sequences. *, amino acids conserved in all sequences; ·, amino acids conserved in at least two sequences.

This fragment has the same size as the transforming vector. These characteristics are diagnostic for a multiple copy integration of the transforming plasmid as tandem arrays. Moreover, the appearance of more than two fragments representing the border sequences of the tandemly integrated plasmid copies (Ts^{-}9-11-1.25),

B

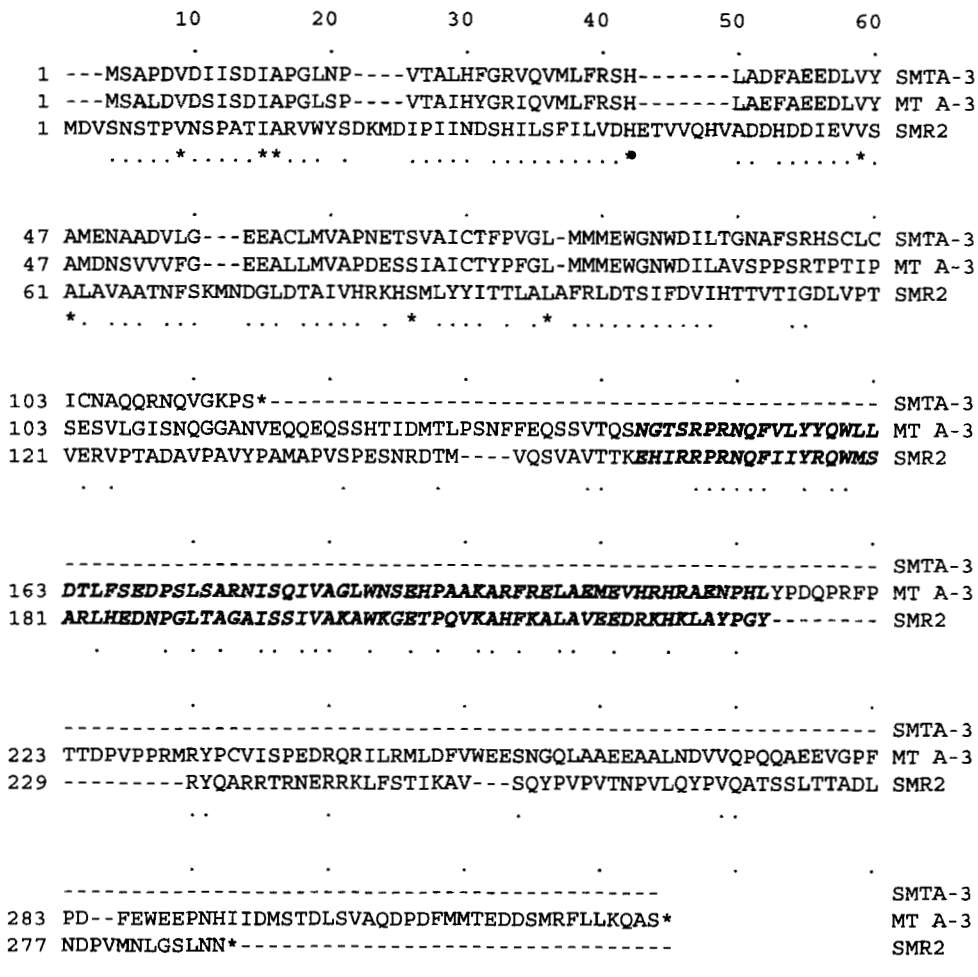

FIGURE 2.—*Continued*

which hybridize with a lower intensity, suggests that multiple copy integration either occurred at several loci or that DNA rearrangements appeared in these transformants. The other three transformants do not contain tandemly arranged multiple plasmid copies but carry either one complete plasmid copy or only a part of it. To discriminate between these two possibilities and to further elucidate the reason why two transformants do not form perithecia at all, a duplicate Southern blot was hybridized with the DIG-labeled insert of plasmid 9-11. Two transformants that are unable to produce perithecia did not give rise to hybridization signals (Figure 4B). On the other hand, one *Cla*I fragment from transformant Ts⁻9.11-1.13 hybridized to the probe. Taken together, it thus appears that the three transformants that are able to produce perithecia do contain the *Smta*-1 sequence of *S. macrospora* whereas the two transformants that do not produce perithecia do not. The latter strains may be the result of plasmid rearrangements, which may have occurred during the transformation process or in a later step that led to the loss of the *Smta-1* sequence. Finally, the data indicated that the number of integrated plasmid copies affects the efficiency of perithecia formation in Podospora transformants.

Functional analysis of mutant derivatives of the mating-type genes: The transformation experiments suggested that the protein encoded by the *S. macrospora Smta-1* gene is the essential factor for the initiation of fruiting body development in *P. anserina mat*− strains, whereas in *P. anserina mat*+ strains, initiation of perithecia development is caused by the polypeptide encoded by the *SmtA-1* gene. To test this hypothesis, we performed an *in vitro* mutagenesis with the two ORFs: *Smta-1* and *SmtA-1*. In plasmid p33-6, the ORF *Smta-1* carries an insertion of 18 bp, leading to the insertion of six additional codons with the deduced hexapeptide sequence Arg-Pro-Asn-Ser-Gly-Asp at amino acid position 99. This insertion is located upstream of the HMG domain, and does not destroy the reading frame. Ts⁻9-11-1.13 and Ts⁻9-11-1.23, expressing the unaltered *Smta-1* gene, and *mat*− transformant Ts⁻p33-6-1.12, expressing the mutant *Smta-1* gene, developed perithecia (Table 3). In contrast, transformants containing plas-

C

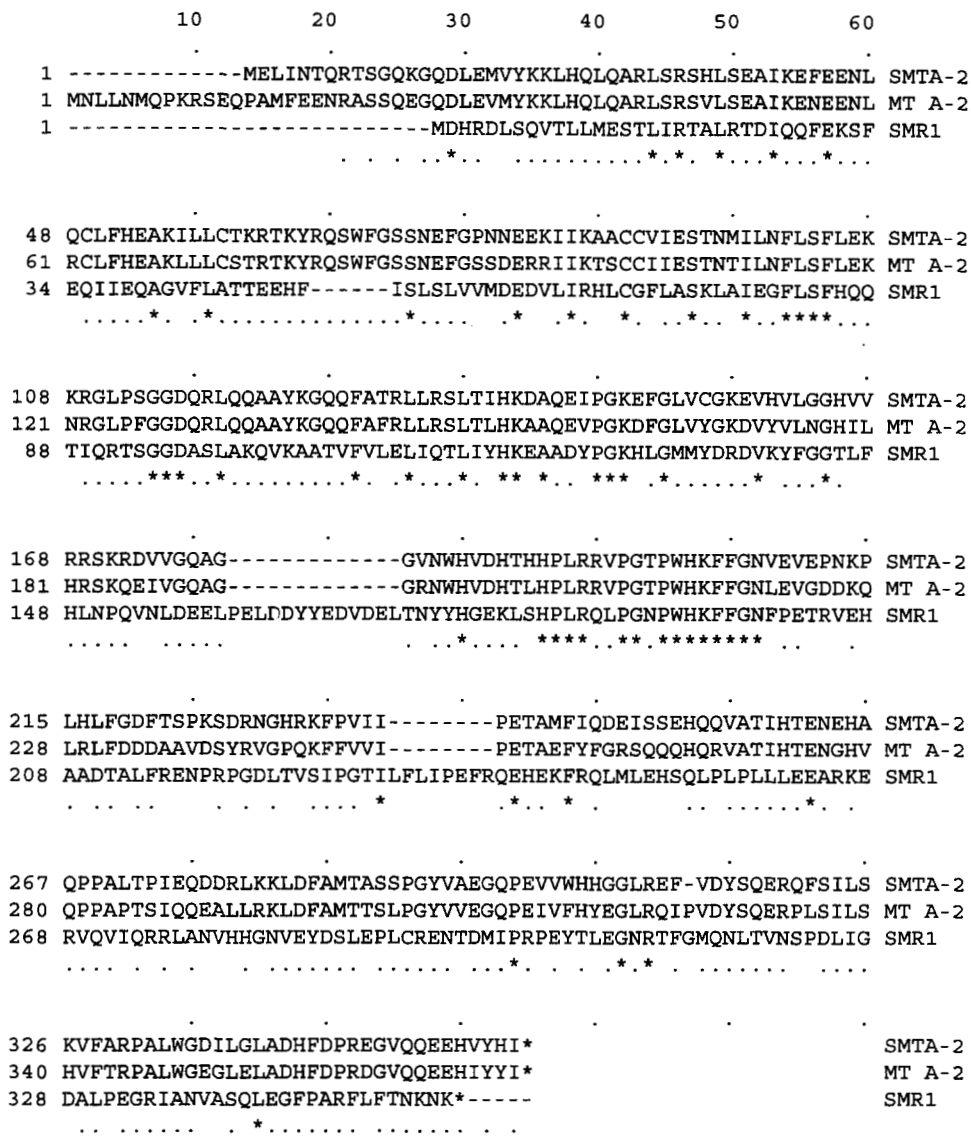

FIGURE 2.—*Continued*

mid p51-4, which carries a mutant *Smta-1* gene with a deletion of 32 bp [a (−1) frameshift that severely truncates the SMTa-1], developed no perithecia (Table 3).

A deletion of 41 bp, leading to a (-1) frameshift at amino acid position 192, was introduced into the *SmtA-1* gene (plasmid p37-74). The mutation inserts a premature stop codon downstream of the α domain. *P. anserina mat+* transformants carrying the frameshift allele of *SmtA-1* express a SmtA-1 protein that lacks 96 amino acids at the C terminus. None of the transformants that received plasmid p37-74 produced fruiting bodies (Table 3). The results obtained with the frameshift constructs indicate that the polypeptide encoded by the unaltered *Smta-1* gene indeed controls fruiting body development in *P. anserina mat−* strains, whereas only the wild-type *SmtA-1* gene is able to induce perithecial formation in homokaryotic *mat+* strains of *P. anserina*.

DISCUSSION

Similarities and differences between homothallic and heterothallic mating types: Here, we report the isolation and sequence analysis of the mating-type locus from the homothallic fungus *S. macrospora*. Sequence analysis revealed four ORFs, *Smta-1, SmtA-3, SmtA-2, SmtA-1*, showing strong similarities to mating-type genes from *N. crassa* and *P. anserina*.

Previous hybridization experiments had shown that *S. macrospora* contains single copy sequences with homology to both *N. crassa A* and *a* idiomorphs (GLASS *et al.* 1990b). Our sequence analysis confirmed the assumption by GLASS *et al.* (1990b) that in *S. macrospora*, *a*-specific and *A*-specific sequences are directly linked and, as shown for the first time, are even contiguous. This result marks the first striking difference between

D

```
          10        20        30        40        50        60
           .         .         .         .         .         .
 1 MSSVDQIVKTFAKLPEGERNAAVNAILAMMPPGPGPVRQIPEPVSQAPAPKKKVNGFMGF SMTA-1
 1 MSGVDQIVKTFADLAEDDREAAMRAFSRMMRRGTEPVRRI-------PAAKKKVNGFMGF MT A-1
 1 MAGINSILQTFEGLGEGDRAETIKVLSDMMREGT--PRQ---------PAKKKVNGFMGY FMR1
   *.....*..**. * *..* ..  . . **. *. . *..        ...*********.

          .         .         .         .         .         .
61 RSYYSPLFSQFPQKARSPFMTILWQHDPFHNEWDFMCSVYSSIRNYLEQSNAQREKKITL SMTA-1
54 RSYYSPLFSQLPQKERSPFMTILWQHDPFHNEWDFMCSVYSSIRTYLEQ------EKVTL MTA-1
50 RSYYSSMFSQLPQKERSPILTTLWQQDPFHKEWDFMCAVYSAIRDQLA------EQNVTL FMR1
   *****..***.***.***..*.***.****.******.***.** .*..     . ..**

          .         .         .         .         .         .
121 QYWLHFAVPDMGVLGRENYLPTLGWDLVTMPNGTIDLMRIAMPLFRKNLQPMNGLCLFTK SMTA-1
108 QLWIHYAVGHLGVIIRDNYMASFGWNLVRFPNGTHDLERTALPLVQHNLQPMNGLCLLTK MTA-1
104 QTWIQFAVTPLGIAPRTGYMEALGWVLTRLDDGTHTLQRMDVPDIRYHLQPMNGLGLFLS FMR1
    * *...**  .*.  * .*.  .** *.. ..**..* * . *. . .*******.*...

          .         .         .         .         .         .
181 CQEGGLQVDNQHLVIAKLSDPSYDMIWFNKRPHYQQR---------------------HA SMTA-1
168 CLESGLPLANPHSVIAKLSDPSYDMIWFNKRPHRQQG---------------------HA MTA-1
164 CLNGGLPIFDPQNIISQLSDPAFDVICINTQVPKIPGTFDTMSGFRQLAKQNPALAMSSL FMR1
    *...**.  ... .*..****..*.*..... ...                        ..

          .         .         .         .         .         .
220 VQADSSELGVSALFPRNHAVAAEADDVTTLQLPHWMQQGDFGTESGYSPQFETLLGSILE SMTA-1
207 VQTDESEVGVSAMFPRNHTVAAEVDGIINLPLSHWIQQGEFGTESGYSAQFETLLDSILE MTA-1
224 FQLPDTDPLIAQGVGMYEFHSVVSQPVQNHGMPPTTVPPMESHSHEDNMDFAKINEAELD FMR1
    .* . .. .... ..... .... . . .. .... ... ........ .*.... ..*.

          .         .         .         .         .         .
280 NGNATSNDSYNMALAMDVPMMGFNGGA*                               SMTA-1
267 NGHASSNDPYNMALAIDVPMMGFNGGA*                              MTA-1
284 AILTMYDTNTNGYIDPNKPQGF*-----                              FMR1
    .. . ... .*.... ..*........
```

FIGURE 2.—*Continued*

the mating type from the homothallic fungus *S. macrospora* and the heterothallic ascomycetes *N. crassa* and *P. anserina*. A homokaryotic individual of the latter two heterothallic filamentous fungi carries mating-type sequences belonging either to the a/*mat*+ or the *A*/*mat*- idiomorph (Figure 5). Recently, it was presumed that the homothallic ascomycete *N. terricola* has an arrangement of *A*- and *a*-specific sequences unlike that found in the mating-type locus of *S. macrospora*. Hybridization analysis revealed that both the *A* and *a* sequences are present on the same chromosome but are probably not closely linked in *N. terricola* (BEATTY *et al.* 1994).

The molecular characterization of the mating-type idiomorphs in *N. crassa* and *P. anserina* showed that both the *a* and *mat*+ idiomorphs contain a single gene relevant to the sexual cycle. A similar situation is found in the Loculoascomycete *C. heterostrophus*, where the MAT-2 idiomorph contains also only a single gene. These genes were called *mta-1*, *FPR1*, and *MAT-2*, respectively (STABEN and YANOFSKY 1990; DEBUCHY and COPPIN 1992; TURGEON *et al.* 1993; Figure 5). Similarly, in *S. macrospora* the region that is homologous to the *a* mating-type idiomorph from *N. crassa* encodes only one gene. Because of its strong amino acid identity of 81.6% to the *N. crassa mta-1* gene, it is called *Smta-1* (Figure 2A). The product of the *N. crassa mta-1* gene appears to be a sequence specific DNA binding protein. The HMG box is sufficient for DNA binding *in vitro* and necessary for mating *in vivo* (PHILLEY and STABEN 1994). The SMTa-1 protein HMG box domain (amino acid position 119–200) is in the same position as in the *N. crassa* MTa-1 polypeptide (Figure 2A) and differs at only six out of 82 amino acids in this region. This similarity implies DNA binding properties for *S. macrospora* SMTa-1. Despite their strong amino acid similarity, the MTa-1 and SMTa-1 proteins differ in length. The *Smta-1* gene encodes a polypeptide that, in comparison with MTa-1, lacks 100 amino acids at the C terminus. Functional analyses *in vivo* had shown that the acidic carboxyl terminal region of the MTa-1 polypeptide contained some essential determinants for mating and for heterokaryon incompatibility (PHILLEY and STABEN

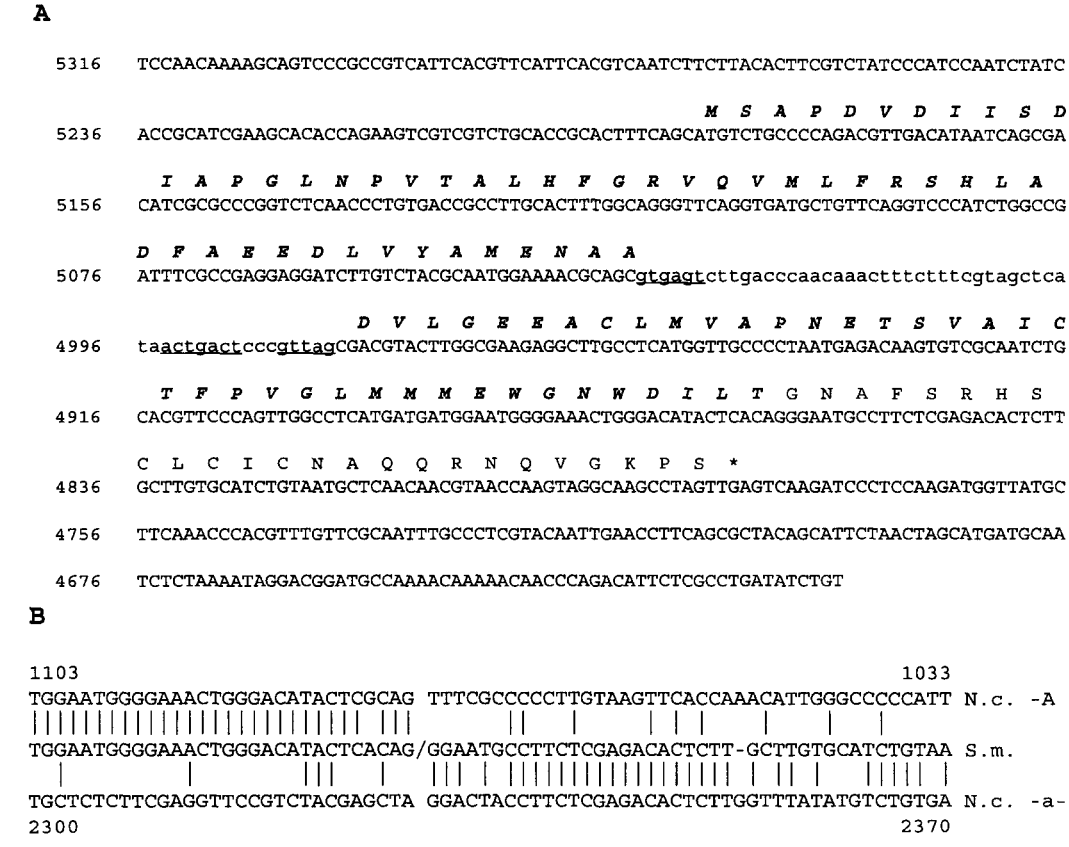

FIGURE 3.—Nucleotide sequences and sequence comparison of the *S. macrospora SmtA-3* mating-type gene. (A) Nucleotide sequence as well as flanking regions of *SmtA-3*. The amino acid sequence of the protein deduced from the cDNAs is shown above the nucleotide sequence. Amino acids homologous to the MTA-3 protein of *N. crassa* are indicated in bold italics. *, the stop codon; the intron-sequence is indicated in lower case letters, and characteristic intron sequences are underlined. The nucleotide sequence is numbered on the left according to the sequence of the *S. macrospora* mating-type locus deposited in the EMBL sequence data base under accession No. Y10616. (B) Comparison of the *S. macrospora* (S.m.) mating-type sequence from position 4890 to 4821 with those from the *N. crassa A* (N.c. -A-) and *a* (N.c. -a-) mating-type idiomorphs as indicated. The drawn line dividing *A*- and *a*-related areas of the *S. macrospora* region is indicated by a slash in the *S. macrospora* sequence.

1994). Heterokaryon incompatibility is irrelevant in homothallic fungi, therefore it is not surprising that the C-terminal region from SMTa-1 differs from that of the Neurospora MTa-1 protein.

In addition to the *Smta-1* gene, three *A*-specific mating-type genes, *SmtA-3*, *SmtA-2* and *SmtA-1*, were identified in the *S. macrospora* mating-type locus. Both the *N. crassa A* and the *P. anserina mat−* idiomorph display a similar structure (Figure 5).

However, in particular, the *SmtA-3* ORF shows different characteristics when compared with the corresponding genes from *N. crassa* (*mtA-3*) and *P. anserina* (*SMR2*). The genomic region encoding the *SMR2* and *mtA-3* genes in *P. anserina* and *N. crassa* (DEBUCHY *et al.* 1993; FERREIRA *et al.* 1996) corresponds to the border region between *A*- and *a*-specific sequences in *S. macrospora* (Figure 5). Therefore, *SmtA-3* has a chimeric character and contains sequences similar to the *A* and *a* mating type idiomorphs from *N. crassa* (Figure 3B). A high degree of identity (89.0%) to the MT A-3 protein of *N. crassa* is only found within the 91 N-terminal amino acids of SMTA-3 (Figure 2B). Within the 25 C-terminal amino acids no significant homology to any mating-type protein and even to any protein in the database could be identified. In contrast to the MTA-3 and SMR2 proteins, a HMG motif, which most probably is involved in DNA binding, is lacking in the putative SMTA-3 protein. The different mating behavior of homothallic and heterothallic fungi might be explained by the chimeric structure of the *SmtA-3* gene and the absence of a HMG domain in the SMTA-3 polypeptide. In *P. anserina*, SMR2, the gene product of the *SmtA-3* homologue, interacts with the FMR1 protein, and the formation of this heterodimer seems to be required for recognition of nuclear identity during the early steps of sexual reproduction (ZICKLER *et al.* 1995). Hybridization studies of mating-type loci from homothallic ascomycetes revealed that the *mtA-3* ORF was conserved in species of Gelasinospora and in *Anixiella sublineata* but not in *N. terricola* (BEATTY *et al.* 1994). However, no sequence data are yet available from the mating-type loci of these homothallic fungi, and it has to be awaited whether the chimeric organization of the *S. macrospora SmtA-3* gene is a general feature of homothallic Sordariaceae.

TABLE 3

Mean diameter of protoperithecia/perithecia from different *Podospora anserina* strains and transformants

Strain/transformant	*mat*-information from *S. macrospora*	Diameter (μm)[a]	Perithecia/protoperithecia >120 μm (%)
700 004: *mat+*[b]	—	42.5 ± 12.3	0.0
700 005: *mat−*[c]	—	47.8 ± 13.4	0.4
700 004 × 700 005[d]	—	91.3 ± 56.5	24.4
Ts^+ D1-1.13[e]	*A*/*a*	82.5 ± 56.5	26.4
Ts^- D1-3.20[f]	*A*/*a*	84.7 ± 64.6	27.6
Ts^+ pB122-1.7[e]	*A*	77.8 ± 40.4	12.0
Ts^+ pB122-1.9[e]	*A*	83.8 ± 52.3	17.2
Ts^+ p26-36-1.17[e]	*A*	91.1 ± 60.6	32.8
Ts^+ p37-74-2.4[e,g]	*A*	45.5 ± 17.7	0.8
Ts^+ pH8-302-1.6[e]	*A*	53.6 ± 19.4	1.2
Ts^- pH8-302-1.5[f]	*A*	44.9 ± 11.2	0.0
Ts^- p9-11-1.13[f]	*a*	73.0 ± 47.6	18.4
Ts^- p9-11-1.23[f]	*a*	78.6 ± 58.9	26.8
Ts^- p9-11-1.9[f]	*a*	51.5 ± 18.6	1.2
Ts^- p9-11-1.5[f]	*a*	54.7 ± 19.5	0.8
Ts^-p33-6-1.12[f,g]	*a*	73.4 ± 71.2	24.0
Ts^- p51-4-1.3[f,g]	*a*	52.3 ± 17.1	0.8

Protoperithecia/perithecia (250) were measured in steps of 5 μm.
[a] Values are means ± SD.
[b] 700 004, *mat+* wild-type strain.
[c] 700 005, *mat−* wild-type strain.
[d] 700 004 × 700 005, wild-type cross.
[e] Ts^+, *mat+* transformant strains.
[f] Ts^-, *mat−* transformant strains.
[g] Strains that carry mutated *S. macrospora* mating-type genes.

The deduced SMTA-2 amino acid sequence shows a strong identity (71.3%) to the *N. crassa* MTA-2 protein. In contrast, the level of amino acid identity between the *S. macrospora* SMTA-2 protein and *P. anserina* SMR1, is fairly low (12.5%). An amphipathic α helix of SMR1, which resembles the transactivating domains of transcriptional activators (GINGER and PTASHNE 1987; DEBUCHY *et al.* 1993), corresponds to a gap in its alignment with the *N. crassa* MTA-2 polypeptide (FERREIRA *et al.* 1996) and with SMTA-2 (Figure 2C). A region of high similarity between the *P. anserina* SMR1 and the *N. crassa* MTA-2 polypeptides (SMR1 positions 183–199, MTA-2 positions 203–219), which has been suggested as a new DNA-binding motif (DEBUCHY *et al.* 1993), could also be identified in SMTA-2 (position 190–206) (Figure 2C). These sequence similarities suggest that SMTA-2, like SMR1 and MTA-2, is involved in postfertilization processes. The effects of SMR1 and MTA-2 on ascosporogenesis are different (GLASS and LEE 1992; ZICKLER *et al.* 1995). In contrast to mutations in *SMR1*, which lead to the production of 100% *mat−* uniparental progeny when crossed with a wild-type tester, *mtA-2* mutants generate biparental progeny when crossed with a wild-type *a* strain (GLASS and LEE 1992; ZICKLER *et al.* 1995; FERREIRA *et al.* 1996). In *S. macrospora,* mating-type functions that depend on the *SmtA-2* gene may involve mechanisms more similar to *N. crassa* than to *P. anserina.*

SmtA-1 shows strong similarities to the *mtA-1* gene from *N. crassa* and the *P. anserina FMR1* gene of the *A* and *mat-* idiomorphs, respectively (Figure 2D). The *SmtA-1* ORF reveals also a region of homology to the single *MAT-1* gene from the MAT-1 idiomorph of *C. heterostrophus* (Figure 5). Like its counterparts in *N. crassa, P. anserina,* and *C. heterostrophus,* the *SmtA-1* ORF encodes a polypeptide similar to the MTα1 mating-type polypeptide of *S. cerevisiae* (SMTA-1 positions 51–103). The region of homology is a DNA-binding motif of ~50 residues, the α1 domain (ASTELL *et al.* 1981; GLASS *et al.* 1990a; DEBUCHY and COPPIN 1992). The C termini of the α1 domain proteins are usually poorly conserved (SAUPE *et al.* 1996), but the C termini from SMTA-1 and MTA-1 show an amino acid sequence similarity of 98% (77% identity) in the 100 C-terminal residues. In contrast to this, a similarity in this region is not detectable between the *P. anserina* FMR1 and the *S. macrospora* SMTA-1 polypeptide. Two regions of dissimilarity in the alignment with MTA-1 and FMR1 (positions 41–47 and positions 110–115, Figure 2D) may indicate a different function of this putative transcriptional regulator in homothallic and heterothallic species of the Sordariaceae.

Mating-type genes from a homothallic are transcriptionally expressed: RT-PCR analyses revealed that *Smta-1, SmtA-3, SmtA-2* and *SmtA-1* were transcriptionally expressed in *S. macrospora.* This is an important hint that the mating-type genes from *S. macrospora* are functional

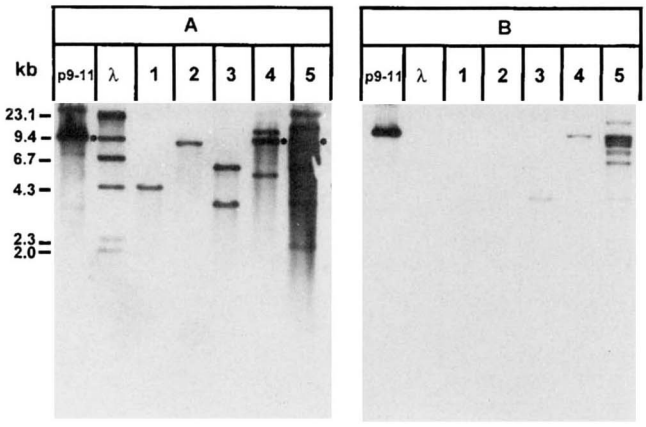

FIGURE 4.—Southern analysis of individual *P. anserina mat*− transformants obtained after transformation with p9-11. The selected transformants differed in their ability to produce perithecia. Genomic DNA isolated from *P. anserina* transformants (lanes 1–5) were hybridized either to DIG-labeled p9-11 (A) or to the DIG-labeled *Eco*RV insert from plasmid p9-11 (B). DNAs were digested with *Cla*I. Lane 1, Ts⁻p9-11-1.3 (no perithecia); lane 2, Ts⁻p9-11-1.5 (no perithecia); lane 3, Ts⁻p9-11-1.24 (few perithecia); lane 4, Ts⁻p9-11-1.13 (many perithecia); lane 5, Ts⁻p9-11-1.25 (many perithecia). Positions and sizes of DNA marker fragments from *Hin*dIII digested phage λ DNA (lane λ) are indicated in the left margin. p9-11 indicates the *Cla*I-digested plasmid p9-11 as a positive control; *, the amplified *Cla*I fragment form the vector molecule.

in homothallic ascomycetes and most probably are, like in heterothallic fungi, involved in fruiting body development and ascosporogenesis. A comparison of the mating-type genes from *N. crassa* and *S. macrospora* shows that the positions of the nine introns identified in the *S. macrospora* genes (*Smta-1*, two introns; *SmtA-3*, one intron, *SmtA-2*, four introns, *SmtA-1* two introns) are perfectly conserved in the two species. The situation is different when the *S. macrospora* genes are compared with the mating-type genes of *P. anserina*, where only the second intron of *Smta-1* and *FPR1*, the first intron of *SmtA-2* and *SMR1*, and the first intron of *SmtA-1* and *FMR1* have conserved positions. The conservation of the intron positions and the results obtained in the above-mentioned sequence-comparison studies suggest a closer evolutionary relationship between *S. macrospora* and *N. crassa* than between *S. macrospora* and *P. anserina*.

The mating-type genes from *S. macrospora* induce perithecial development in a heterothallic fungus: A functional characterization of the mating-type genes was carried out using a transformation assay in the heterothallic fungus *P. anserina*. The transformation studies showed that *S. macrospora* mating-type genes induced perithecial development in *P. anserina*. *N. crassa* mating-type genes can provide fertilization functions in *P. anserina* strains, and, reciprocally, the introduction of *P. anserina* mating-type genes confer mating activity in *N. crassa* (ARNAISE *et al.* 1993). In addition, the mating-type specific homologues of *C. heterostrophus* (Figure 5), a heterothallic member of the Loculoascomycete, are also functional when expressed in *N. crassa* and *P. anserina* (CHRISTIANSEN *et al.* 1993). These results indicate that mating-type products are interchangeable with respect to the fertilization not only between heterothallic ascomycetes but even between homothallic and heterothallic fungi. A functional conservation of vegetative incompatibility and postfertilization functions was not found (ARNAISE *et al.* 1993). The *mtA-1* gene of the homothallic ascomycete *N. africana* functions as a mating-activator and confers mating-type associated vegetative incompatibility in *N. crassa* (GLASS and SMITH

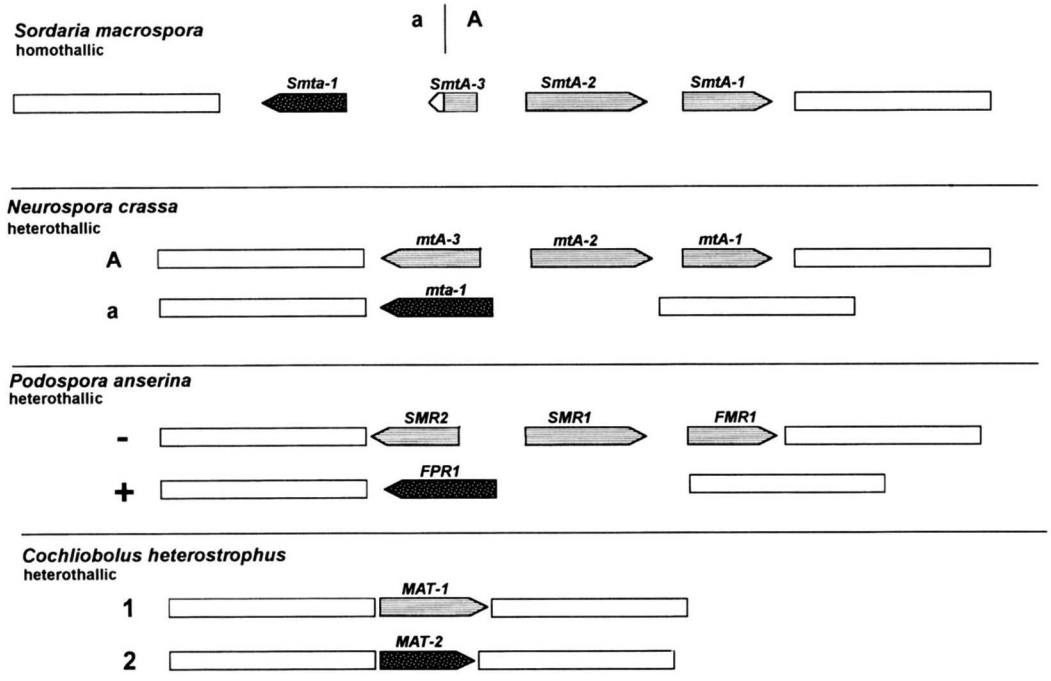

FIGURE 5.—Comparative genetic map of mating-type loci from *S. macrospora* and three heterothallic ascomycetes. Striped and spotted arrows indicate position and orientation of the open reading frames. Boxes correspond to left and right borders of the mating-type regions.

1994). Our transformation studies indicate no conservation of postfertilization functions of the mating-type products from *S. macrospora* and *P. anserina*. *P. anserina* transformants produced perithecia that differed slightly in their morphology when compared with those from a wild-type cross but produced no ascospores. Southern blot analysis revealed that the *S. macrospora* mating-type genes integrated at ectopic genomic positions in the *P. anserina* transformants. Crossing and selfing experiments with *C. heterostrophus*, *N. crassa* and *P. anserina* transgenic strains carrying both introduced and resident mating-type genes have demonstrated the necessity of mating-type transgenes for fruiting body formation, but although plentiful perithecia are formed, ascospore production is extremely low (GLASS *et al.* 1988; PICARD *et al.* 1991; COPPIN *et al.* 1993; TURGEON *et al.* 1993, 1995). The absence of full fertility is apparently due to interference of the resident gene with the function of the transgene. No interference is found when a transgene is expressed in a strain not containing a resident mating-type locus (PICARD *et al.* 1991; COPPIN *et al.* 1993; WIRSEL *et al.* 1996); such strains would be ideal recipients in which to test *SmtA-2* and *SmtA-3* function. From the genetic and functional analysis in the heterothallic ascomycetes, it was presumed that the products of *Smta-1* and *SmtA-1* homologues function as transcriptional activators and confer mating identity. It was thus suggested that genes encoding mating-specific pheromones and receptors, and genes involved in the fusion of cells with opposite mating type could be potential target genes for the mating-type products (ARNAISE *et al.* 1993). Such pheromone signaling pathways have already been proved for the mating-type systems in yeast and in basidiomycetes (for review, see HERSKOWITZ 1989; WENDLAND *et al.* 1995; KAHMANN and BÖLKER 1996). However, homothallic fungi do not need to sense the presence of cells of opposite mating type, and therefore the products of homothallic mating-type genes presumably do not activate pheromone and receptor genes.

An interesting aspect concerning target genes for mating-type products comes from genetic analysis of the mutant *vacua* from *P. anserina*, which produces homokaryotic, sterile perithecia that contain only proliferating paraphyses (ESSER and GRAW 1980). The *vacua* gene is not linked to the mating-type genes, which block fertilization in homokaryons. A mutated *vacua* gene causes a phenotype that is very similar to the one found in *P. anserina* transformants carrying the *S. macrospora* mating-type genes. It may therefore be suggested that the *vacua* gene is one of the target genes that are activated by the *S. macrospora* mating-type products.

In conclusion, the sequence and functional analysis of the mating-type locus from the homothallic ascomycete *S. macrospora* reveals a striking conservation of mating-type sequences and functions in homothallic and heterothallic fungi. On the other hand, these results raise interesting questions concerning the differences between sexual development in homothallic and heterothallic ascomycetes. In the future, these questions must be further explored by functional analysis of the mating-type genes and by screening the target genes of homothallic and heterothallic mating-type products.

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