Co-expression of the Mating-Type Genes Involved in Internuclear Recognition Is Lethal in *Podospora anserina*

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ABSTRACT

In the heterothallic filamentous fungus *Podospora anserina*, four mating-type genes encoding transcriptional factors have been characterized: *FPR1* in the *mat*+ sequence and *FMR1*, *SMR1*, and *SMR2* in the alternative *mat*− sequence. Fertilization is controlled by *FPR1* and *FMR1*. After fertilization, male and female nuclei, which have divided in the same cell, form *mat*+/*mat*− pairs during migration into the ascogenous hyphae. Previous data indicate that the formation of *mat*+/*mat*− pairs is controlled by *FPR1*, *FMR1*, and *SMR2*. *SMR1* was postulated to be necessary for initial development of ascogenous hyphae. In this study, we investigated the transcriptional control of the *mat* genes by seeking *mat* transcripts during the vegetative and sexual phase and fusing their promoter to a reporter gene. The data indicate that *FMR1* and *FPR1* are expressed in both mycelia and perithecia, whereas *SMR1* and *SMR2* are transcribed in perithecia. Increased or induced vegetative expression of the four *mat* genes has no effect when the recombined gene is solely in the wild-type strain. However, the combination of resident *FPR1* with deregulated *SMR2* and overexpressed *FMR1* in the same nucleus is lethal. This lethality is suppressed by the expression of *SMR1*, confirming that *SMR1* operates downstream of the other *mat* genes.

THE mating-type locus of the filamentous ascomycete *Podospora anserina* appears to be a master regulatory locus, mainly controlling self-nonself recognition between cells at fertilization and between nuclei after fertilization. Four genes assumed to encode transcriptional factors were characterized (see Figure 1): *FPR1* in the *mat*+ haplotype and *FMR1*, *SMR1*, and *SMR2* in the alternative *mat*− haplotype corresponding to completely different DNA sequences (Debuchy and Coppin 1992; Debuchy *et al.* 1993). At fertilization, *FPR1* and *FMR1* determine, respectively, mat+ and mat− mating specificity, mediating recognition between male gametes and female organs (Coppin *et al.* 1993) probably through a pheromone/receptor system as in yeasts (reviewed in Herskowitz 1988). After fertilization, all four *mat* genes control an initial stage of perithecial development that requires recognition between *mat*+ and *mat*− nuclei (Zickler *et al.* 1995). In fact, *mat*+ and *mat*− nuclei of female and male origin do not fuse immediately after fertilization but proliferate in syncitial conditions; afterwards, pairs of nuclei of opposite mating type migrate to specialized hyphae, the ascogenous hyphae, which divide in an intricate manner: they form hook-shaped cells called croziers in which the dikaryotic (*mat*+/*mat*−) state is maintained. Nuclear fusion occurs in the apical cell of the crozier and is followed by meiosis and formation of asci with a strict 1:1 ratio of *mat*+ and *mat*− nuclei (see Raju and Perkins 1994 and Thompson-Coffe and Zickler 1994). The success of the sexual process relies on the proper association of *mat*+ and *mat*− nuclei in the ascogenous hyphae and requires that nuclei of each parent recognize each other as different. This process will be referred to hereafter as internuclear recognition (IR). Mutations in *FPR1*, *FMR1*, or *SMR2* were shown to lead to aberrant progeny with non-Mendelian segregation and this phenotype was interpreted as resulting from improper recognition between nuclei (Zickler *et al.* 1995). *FPR1* was characterized as the *mat*+ gene involved in IR and *FMR1*/*SMR2* as the *mat*− genes involved in IR (Zickler *et al.* 1995; Arnaise *et al.* 1997). *SMR1* is only required for postfertilization development, but unlike *FMR1*, *SMR2*, and *FPR1* it does not confer any mating-type identity to nuclei. Crosses with transgenic strains indicate that *SMR1* can fulfill its function either in the *mat*− parent or in the *mat*+ parent or even in both parents (Arnaise *et al.* 1997). Consequently, although *SMR1* lies at *mat* locus, it does not behave as a mating-type gene *sensus stricto*. In crosses with *SMR1* deletion mutants, perithecia are blocked very early in their development and no progeny are recovered (Arnaise *et al.* 1997). Indirect arguments mainly based on epistatic relationships between mutations in *mat* genes (S. Arnaise, personal communication) suggest that *SMR1* acts downstream of IR genes for initial development of the ascogenous hyphae after nuclear pairing. Its definite function is still unknown.

IR is a brief event, occurring in the early stage of fruiting-body development. That implies evident diffi-

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culties in observation and analysis. On the assumption that vegetative expression of mating-type genes may mimic IR in the mycelium and aid in its analysis, we investigated the control of expression of mating-type genes in wild-type strains and the effects of deregulated IR genes (*FMR1*, *SMR2*, *FPR1*) and *SMR1* during the vegetative phase. Expression studies showed that *FMR1* and *FPR1* are active during both the vegetative and sexual reproduction phase of *P. anserina*, while *SMR1* and *SMR2* are not vegetatively transcribed. Deregulated *SMR2* and *SMR1* and vegetatively overexpressed *FMR1* transgenes have been associated in various combinations by crossing. The association of *FMR1* and *SMR2* was found to lead to ascospore lethality in *mat*+ genetic context. Germination of ascospores was shown to be restored by introducing a vegetatively expressed *SMR1* transgene. In the frame of the functional model of the mating types, we will discuss how these results can be interpreted and exploited for further investigations.

MATERIALS AND METHODS

P. anserina: **genetic analysis, strains, transformation:** The ascus of *P. anserina* normally contains four ascopores that develop around two nonsister nuclei after a single postmeiotic mitosis. However, 2 to 5% of asci contain five ascospores, two of which are smaller and uninucleate, yielding homokaryotic mycelium. Tetrad analysis is routinely performed on five-spored asci. The Δ*mat* strain used in the study is derived from a *mat*+ strain deleted for the *mat* locus (Coppin *et al.* 1993). The *leu1-1 mat*− (*SMR2::ura5*) strain was obtained from a *mat*− strain in which the resident *SMR2* gene was disrupted, and the *mat*− (*SMR1::ura5*) strain carries a *SMR1* disruption at the resident *mat*− locus (Arnaise *et al.* 1997). Transformation was performed as previously described (Picard *et al.* 1991). When necessary, phleomycin (Cayla, France) or hygromycin (Roche Diagnostics, Meylan, France) was added to protoplast regeneration medium at a concentration of 5 μg/ml and 100 μg/ml, respectively. Segregation of antibiotic resistance in the sexual crosses was scored on minimal medium containing either 20 μg/ml of phleomycin or 75 μg/ml hygromycin.

Bacterial strains, plasmids, and plasmid constructions: Cloning and plasmid preparations were performed with *Escherichia coli* HB101 (Boyer and Roulland-Dussoix 1969). The plasmid pUL contains the *leu1* gene (Turcq 1989) of *P. anserina* on a 2.1-kb *Hin*dIII-*Pst*I fragment in the vector pUC 18 (Yanisch-Perron *et al.* 1985). The 5.7-kb *Pst*I-*Pst*I fragment, which contains the entire *mat*− sequence, has been cloned in pUL to give pULP (Debuchy *et al.* 1993). The pULP-68 plasmid was derived from pULP by the deletion of *SMR2*. Plasmid pSUT12 contains *SMR1* and *SMR2* on a 3.4-kb *Cla*I-*Xba*I fragment isolated from pULP and cloned into pUT703 (Calmels *et al.* 1991). Plasmid pUT703 harbors the *ble* gene under the control of the *gpd* promoter of *Aspergillus nidulans* (Punt *et al.* 1988) and determines phleomycin resistance upon transformation in fungi. Plasmid pCBSMR2 is based on pCB1004, carrying the *hph* gene conferring resistance to hygromycin upon transformation in fungi (Carroll *et al.* 1994); it bears *SMR2* on a 2.5-kb *Eco*RI-*Pst*I fragment derived from pULP (Debuchy *et al.* 1993). Plasmid pucES+ contains the 6.3-kb *Eco*RI-*Sal*I fragment with the complete *mat*+ information (Debuchy *et al.* 1993). A restriction map of the *mat*+ and *mat*− loci is presented in Figure 1A.

Plasmids pPUTUL, pFUTUL, pSUTUL1, and pSUTUL2 contain the *ble* gene (Drocourt *et al.* 1990) under the control of the translation initiation and upstream sequence of *FPR1*, *FMR1*, *SMR1*, and *SMR2*, respectively (Figure 1A). The *ble* gene, conferring resistance to phleomycin, was prepared using the pUT703 plasmid (Calmels *et al.* 1991). All the plasmids were based on pUL. Plasmid pPUTUL contains an in-frame fusion of the first 10 residues of FPR1, preceded by 1 kb of upstream untranslated region (UTR) with the *ble* gene at the *Nco*I site. The *Nco*I site has been introduced in *FPR1* by amplification from KSRIRV (Debuchy and Coppin 1992) with a reverse primer and 5′-CGCCATGGAGAAGGCTTCAAAATTGAA GGC-3′ followed by digestion with *Nco*I. pFUTUL was constructed by ligation of the 1.02-kb *Eco*RI-*Stu*I *mat*− fragment encoding the initial 13 residues of FMR1 with the *ble* gene. pSUTUL1 was constructed by ligation of the 0.59-kb *Eco*RI-*Bgl*II fragment encoding the first four residues of SMR1 with the *ble* gene. The *Eco*RI-*Bgl*II fragment was prepared from pULP-68. pSUTUL2 was constucted by ligation of a 0.74-kb *Bgl*II-*Eco*RV *mat*− fragment encoding the first 22 residues of SMR2 with the *ble* gene. The in-frame fusion between the *mat*− gene and the *ble* gene in pFUTUL, pSUTUL1, and pSUTUL2 has been reexamined by DNA sequencing. Plasmid pFLUT is a pUL, in which the *ble* coding sequence was cloned, ligated to the 60-bp *Cla*I-*Stu*I *mat*− fragment. This fragment contains 21 bp upstream of the coding sequence of *FMR1* and encodes the first 13 residues of *FMR1* in frame with the *ble* coding sequence.

Plasmids pGFMR1, pGSMR1, and pGSMR2 (Figure 1A) are based on the pUL vector and contain the *A. nidulans gpd* promoter on a 2.3-kb *Eco*RI-*Nco*I fragment of pUT703 (Calmels *et al.* 1991) fused to *mat*− genes. Plasmid pGFMR1 was constructed by ligation of the *gpd* promoter with the 1.35-kb *Cla*I-*Xba*I *mat*− fragment, resulting in a promoter fusion 21 bp upstream of the translation start of *FMR1*. pGSMR1 has been constructed by the ligation of the *gpd* promoter with the 1.35-kb *Nco*I-*Pst*I fragment that contains the *SMR1* gene at the start of the initiation codon. This fragment was obtained by amplification using the 48827 [5′-CCCCCCATGGACCACCGA GATCTATCC-3′] and 48829 [5′-GGGGCTGCAGGATCATC TCC-3′] primers on pULP. Plasmid pGSMR2 was constructed using the ligation of the *gpd* promoter with a 1.04-kb *Nco*I-*Pst*I fragment containing the *SMR2* gene that starts at the initiation codon. This fragment was obtained by amplification with the 46303 (5′-CCCCCCATGGATGTCTCCAACTCCAC-3′) primer and reverse primer on pULP followed by enzymatic digestion with *Nco*I and *Pst*I. The *SMR1* and *SMR2* genes in pGSMR1 and pGSMR2 were sequenced and examined for absence of mutation. Plasmid pGFPR1 was obtained from pLFMPR1, which contains a 5′ truncated *FPR1* gene on a 2.99-kb *Ava*II-*Eco*RI *mat*+ fragment fused to a 1.02-kb *Eco*RI-*Stu*I *mat*− fragment encoding the first 13 residues of FMR1. The *FMR1::FPR1* gene fusion was under the control of the 5′ UTR of *FMR1*. This gene fusion confers a *mat*+ phenotype similar to wild-type *mat*+ phenotype (data not shown). The 3.06-kb *Cla*I-*Eco*RI fragment of pLFMPR1, containing the *FMR1::FPR1* fusion and 22 bp upstream of the initiation point, was fused with the *gpd* promoter to yield pGFPR1. Plasmids pPaFMR1 and pPgFMR1 contain *FMR1* under the control of its 5′ UTR and of the *gpd* promoter of *A. nidulans*, respectively. These plasmids are based on pPable, which contains the *ble* gene under control of the *gpd* promoter of *P. anserina* as selective marker for transformation into *P. anserina*. Plasmid pPable was constructed by ligation of a 0.35-kb *Eco*RI-*Nco*I fragment containing the minimal *P. anserina gpd* promoter prepared from plasmid pRP81-1 (Ridder and Osiewacz 1992), with

the 0.7-kb *Nco*I-*Hin*dIII fragment of pUT703 (Calmels *et al.* 1991) containing the *ble* gene in Bluescript KS digested with *Eco*RI and *Hin*dIII. Plasmid pPaFMR1 was generated by cloning the 2.3-kb *Eco*RI-*Xba*I fragment containing the entire *FMR1* coding sequence and its 5′UTR (Debuchy and Coppin 1992) in pPable. Plasmid pPgFMR1 was generated by cloning in pPable the 3.6-kb *Eco*RI-*Xba*I fragment encompassing the *gpd::FMR1* fusion prepared from pGFMR1.

Determination of the phleomycin resistance of the transformants carrying the *5′mat::ble* gene fusion: The *5′mat::ble* fusions cloned in the pUL plasmid were introduced into the *leu1-1 mat*− strain. Transformants (leu$^+$) were then tested on minimal medium containing 20 μg/ml phleomycin. They were considered resistant if growth was observed after 3 days of incubation, since growth of the wild-type strain was totally abolished during this period. A total of 30 to 100 primary (leu$^+$) transformants obtained with each fusion were tested. The phleomycin phenotype was then more accurately determined, using two or three purified transformants issued from crosses of selected primary transformants with a *leu1-1 mat*+ strain. Since the transformants also contained *leu1-1*, the segregation of the integrated transforming vector was easily scored as (leu$^+$) phenotype, independent of expression of the *5′mat::ble* fusion. In these transformants, the integrity of the fusion transgene was ascertained by PCR analysis. The minimal inhibitory concentration (MIC) of phleomycin was determined on *mat*+ and *mat*− progeny carrying the construct. An identical MIC was obtained for strains of opposite mating type. At 10 μg/ml phleomycin, growth of the wild-type strain was inhibited for ~6 days; a residual growth was then observed. The time lag before residual growth was increased when phleomycin was used at 20 μg/ml, and some implants showed zero growth. Transformants with different levels of phleomycin resistance were recovered, depending on the construct introduced. MIC higher than 100 μg/ml phleomycin were not tested.

Genetic analysis of the deregulated *mat* gene associations: First, each *mat*+ *SMR2* transformant containing a constitutively transcribed ectopic *SMR2* gene was crossed with the *mat*− *GFMR1-5* transformant carrying the pGFMR1 plasmid (*gpd::FMR1*, *leu1*). At least 18 five-spored asci from each cross were submitted to genetic analysis and screened for sexual phenotype (mating type and self-fertilization) and hygromycin resistance. Second, each *SMR2* transformant was crossed with transformants carrying the pPgFMR1 plasmid (*gpd::FMR1*, *ble*) or the pPaFMR1 plasmid (*FMR1*, *ble*). At least 20 five-spored asci from each cross were screened for sexual phenotype (mating type and self-fertilization) and hygromycin and phleomycin resistance. In such crosses involving three genetic loci—the mating-type locus, the integration locus of the (*SMR2*, *hph*) transgenes, and the integration locus of the (*gpd::FMR1*, *leu1*, or *ble*) transgenes (provided that these are not genetically linked)—the eight genotypes of homokaryotic progeny listed in Tables 3 and 4 are expected to be equivalent. When a phenotypic class is lacking, tetrad analysis allows us to determine whether it corresponds to ascospores that have not germinated, genotypes of which can possibly be deduced from segregation of the genetic markers in the remaining viable ascospores of the same ascus.

Construction of *mat*+ *SMR2 gpd::FMR1 gpd::SMR1* strain: The *mat*+ *GSMR1-4* and *mat*+ *GSMR1-5* were crossed with the *mat*− *SMR2-19 GFMR1-1* and *mat*− *SMR2-19 GFMR1-2* strains. In the four crosses, 15 five-spored asci were submitted to genetic analysis. In three crosses an additional sample of 60 to 100 homokaryotic ascospores from five-spored asci were also analyzed. The segregation of the *gpd::SMR1* in *mat*+ progeny was scored by ability to restore fertility in sexual cross with the *mat*− (*SMR1::ura5*) mutant. Since no simple functional test for *mat*− progeny was possible, when necessary the presence of the *gpd::SMR1* transgene was established by PCR analysis.

DNA procedures: Genomic DNA of transformants was prepared as described previously (Coppin-Raynal *et al.* 1989). Minipreparations of DNA were done from cultures grown on a cellophane disk placed on agar minimal medium and recovered by scraping with a sterile spatula.

To confirm the genotype of transgenic progeny from sexual crosses, the presence of the *mat* transgenes was tested by PCR analysis. The position of pairs of primers is indicated in Figure 1B, and their sequence is as follows: *FPR1*: E3 [5′-GTCACTGG AACACACTCAAG-3′]; F10 [5′-TTGACCGAAGATTTGGGC-3′]. *FMR1*: 267352 [5′-GGCGGGAATCAACAGTATTTTGC-3′]; 2544 [5′-CATCCAAGGGCTTCCATGTA-3′]. *SMR1:* 247109 [5′-CGCGCATATAATGAATATCACGG-3′]; 7317 [5′-CCCTCCAACTGAGATGCCAC-3′]; *SMR2*: 246738 [5′-GGAT GTCTCCAACTCCACTC-3′]; 3293 [5′-CGTTGAGATCCGCG GTGGTC-3′] .

To analyze the structure of the integrated *5′mat::ble* fusions, PCR amplifications were performed using the ble2 primer [5′-CACGAAGTGCACGCAGTT-3′], localized close to the stop codon in the *ble* gene, in association with a primer specific to the 5′ UTR of the concerned *mat* gene: 573 [5′-CTAATAAGAA TAATGTAATG-3′], which is 540 nucleotides upstream of *FMR1* start codon, 246738, close to the *SMR2* start codon, the reverse primer flanking the *5′-SMR1* sequence in pSUTUL1.

The structure of the integrated *gpd::mat* fusions was analyzed using the 39048 primer [5′-CCATCCTTCCCATCCCTTAT TCC-3′] localized in the *gpd* promoter 100 nucleotides upstream of the initiation codon in association with a primer localized at the 3′ end of the relevant *mat* gene.

Standard procedures for Southern blotting on Hybond N nylon filters (Amersham, France) were used. The probes were prepared using a random primer labeling kit (Roche Diagnostics).

RNA extraction: To prepare RNA from mycelium, fungal cultures were made on a cellophane disk placed on agar minimal medium. After 2 days at 27°, the mycelium was recovered and transferred to a microcentrifuge tube. To prepare RNA from perithecia, cultures of *mat*+ and *mat*− strains were fertilized, respectively, with *mat*− and *mat*+ microconidia on separate petri dishes. Two hundred perithecia samples were collected 3 days after fertilization (production of mature ascospores begins on the fourth day). Perithecia were crushed with a conical grinder in 4 m guanidium thiocyanate, 50 mm TRIS HCl pH 8, 10 mm EDTA pH 8, 2% *N*-lauroylsarcosine (sodium salt), and 1% β-mercaptoethanol. The suspension was treated three times with phenol-chloroform (1:1) and nucleic acids were precipitated with 1 volume of isopropanol. After centrifugation the pellet was resuspended in water. LiCl was added to a final concentation of 2 m, the solution was centrifuged, and the pellet was resuspended in water; sodium acetate pH 5.2 was added to a final concentration of 0.3 m and total RNA precipitated with 2 volumes of ethanol and recovered by centrifugation. The RNA pellet resuspended in water was purified on a RNeasy Plant minikit (QIAGEN, Hilden, Germany) according to the manufacturer's indications. Contaminating DNA was eliminated by Dnase digestion or centrifugation on a CsCl2 cushion. Complete degradation of DNA was ascertained by PCR reaction seeking the internal transcribed sequences of RNA ribosomal genes, with one primer [5′-CCGTTGGTGAACCAGCGGAGGGATC-3′] localized at the end of the 18S gene and the other [5′-TCCGCTTAT TGATATGCTTAAG-3′] at the beginning of the 28S gene. For quantitative competitive RT-PCR, RNA were purified with High Pure RNA kit (Roche Diagnostics).

The reverse transcriptase polymerase chain reaction

method (RT-PCR): Two micrograms of total RNA were used for RT-PCR with the Titan one Tube RT-PCR Kit (Roche Diagnostics) according to the manufacturer's specifications. The following pairs of primers were used (DNA sequence of some primers is cited in DNA procedures): *FPR1*: E3/2551 [5′-GATCTCAGAAGATCGACGAGG-3′]; *FMR1*: 267352/2544; *SMR1* 247109/7317; and *SMR2* 246738/3293. The *gpd::SMR1* transcript was sought using the 2302 primer [5′-GATTGACCTGGGGGTTGAGG-3′] localized downstream of the first intron in association with the *gpd* specific primer 39048. The localization of the primers is shown in Figure 1B.

QC-RT-PCR: Quantification of *FMR1* mRNA was done by competitive RT-PCR (reviewed in Freeman *et al.* 1999). The competitive template for *FMR1* was prepared according to the double-cut method (McCulloch *et al.* 1995) in which both competitive and target molecules contain a unique restriction enzyme site. Any heteroduplexes will remain uncut and separate from the competitor and target. The competitive molecule was prepared by amplification of two overlapping fragments from *FMR1* cDNA. One fragment resulted from the amplification with primer 267352 and IFMR1Hha [5′TTCTTC TTGGCGGGCTGACGCGGTGTGCCTTCCCG-3′] and the second fragment was obtained with primers FMRHha [5′-AGC CCGCCAAGAAGAAGGTCAACGGTTTCATGCGC-3′] and 2544. Overlap extension of these two fragments, followed by PCR with 267352 and 2544, allows us to prepare the competitive molecule that differs from *FMR1* cDNA by the loss of a *Hha*I site at 300 bp from primer 2544 and a new site *Hha*I at 250 bp from primer 2544. The competitive DNA was cloned into pGEM-T (Promega, Madison, WI) to produce pGMFMR1dH. Amplifications were performed with primers 267352 and 2544, the PCR products were digested with *Hha*I, and bands at 300 bp (target) and 250 bp (competitor) were compared for fluorescence on a 2% agarose gel. Target and competitor DNA molecules have been checked for identical amplification kinetic. Competitor RNA was prepared from pGMFMR1dH by transcription with T7 RNA polymerase after linearization with *Sal*I. The RNA was purified with the High Pure RNA kit (Roche Diagnostics) and its integrity was checked by gel electophoresis. To quantitate *FMR1* mRNA, 10-fold serial dilutions ranging from 1 femtomole to 0.001 attomole of competitive RNA and 200 ng of total RNA were added to each tube. After RT-PCR (Titan One Tube, Roche Diagnostics) for 30 cycles, the PCR products were analyzed as indicated above.

RESULTS

Search for the *mat* transcripts in vegetative and sexual phases: To determine if the *mat* genes are differentially transcribed throughout the life cycle of *P. anserina*, total RNAs were isolated from growing *mat*+ and *mat*− mycelial cultures and from perithecia of *mat*+ × *mat*− crosses. *Mat* genes encode regulatory proteins and only low levels of transcripts were expected. In fact, we were unable to detect *mat* mRNA on Northern blots. Therefore, we used the highly sensitive RT-PCR technique. Aliquots of each RNA preparation were used as templates for reverse transcription of RNA, followed by DNA amplification with primers specific for each *mat* gene. The primers indicated in Figure 1B were chosen such that they allowed amplification of a cDNA spanning one or more introns. A PCR product with the expected size for *FPR1* and *FMR1* cDNA was obtained in reactions performed with RNA extracted from either *mat*+ or *mat*− mycelial cultures, respectively. No mature or primary *SMR1* and *SMR2* transcripts were detected in RT-PCR reactions from *mat*− mycelial cultures, although several RNA preparations were tested with different pairs of primers. The cDNA of the four *mat* genes were detected in RT-PCR reactions performed on RNA from 3-day perithecia (data not shown). The fragments were cloned and DNA sequencing demonstrated that they actually corresponded to cDNA with proper intron splicing (Debuchy *et al.* 1993; R. Debuchy, unpublished data).

Transcriptional activity of the 5′ UTR of the *mat* genes during vegetative growth: *FMR1* and *FPR1* RNA detected in whole mycelium extracts can be attributed to contamination of the extracts by sexual organs (protoperithecia or microconidia) or to the expression of *FMR1* and *FPR1* in vegetative hyphae. To test the expression of *FMR1* and *FPR1* in vegetative hyphae, we constructed fusions between *FPR1* and *FMR1* and the *ble* reporter gene (materials and methods). Fusions of *SMR1* and *SMR2* with *ble* were also tested to confirm the negative results obtained in RT-PCR assays. Expression in vegetative hyphae could be easily measured by determining the resistance level to phleomycin conferred by the fusions (materials and methods). The constructs were made in the pUL transformation plasmid containing the *leu1* gene as selective marker. The 5′ UTR and the origin of the coding region of *FPR1* (*mat*+), *FMR1*, *SMR1*, and *SMR2* (*mat*−) were fused in-frame with the entire coding sequence of the *ble* gene, leading to pPUTUL, pFUTUL, pSUTUL1, and pSUTUL2 plasmids, respectively (Figure 1A and Table 1). The in-frame fusions were reexamined by DNA sequencing.

Two series of controls were performed. First, we determined if the 5′ UTR sequences were or were not competent to promote transcription of their native gene. Therefore, each *mat* gene with the upstream sequence used for the fusion with *ble* was introduced by transformation in the suitable recipient and was shown to be functional in assays similar to those described for the *gpd::mat* fusions (see below). Second, as plasmids mainly integrate by heterologous recombination in *P. anserina*, we determined the frequency of downstream integration of a promoter-like sequence. For this control, we used the pFLUT plasmid containing the *ble* coding region fused to a plasmid sequence assumed to be devoid of promoter activity. Data on the transformation experiments are presented in Table 1. One phleomycin-resistant transformant among the 60 tested (leu^+) transformants was obtained with the pFLUT control plasmid. Insertion of the *ble* gene downstream of a genomic promoter-like sequence expressed in the mycelium can therefore be considered as a rare event in comparison with the high frequency of phleomycin-resistant transformants recovered upon transformation with pPUTUL, pFUTUL, and pSUTUL2. Phleomycin-resistant trans-

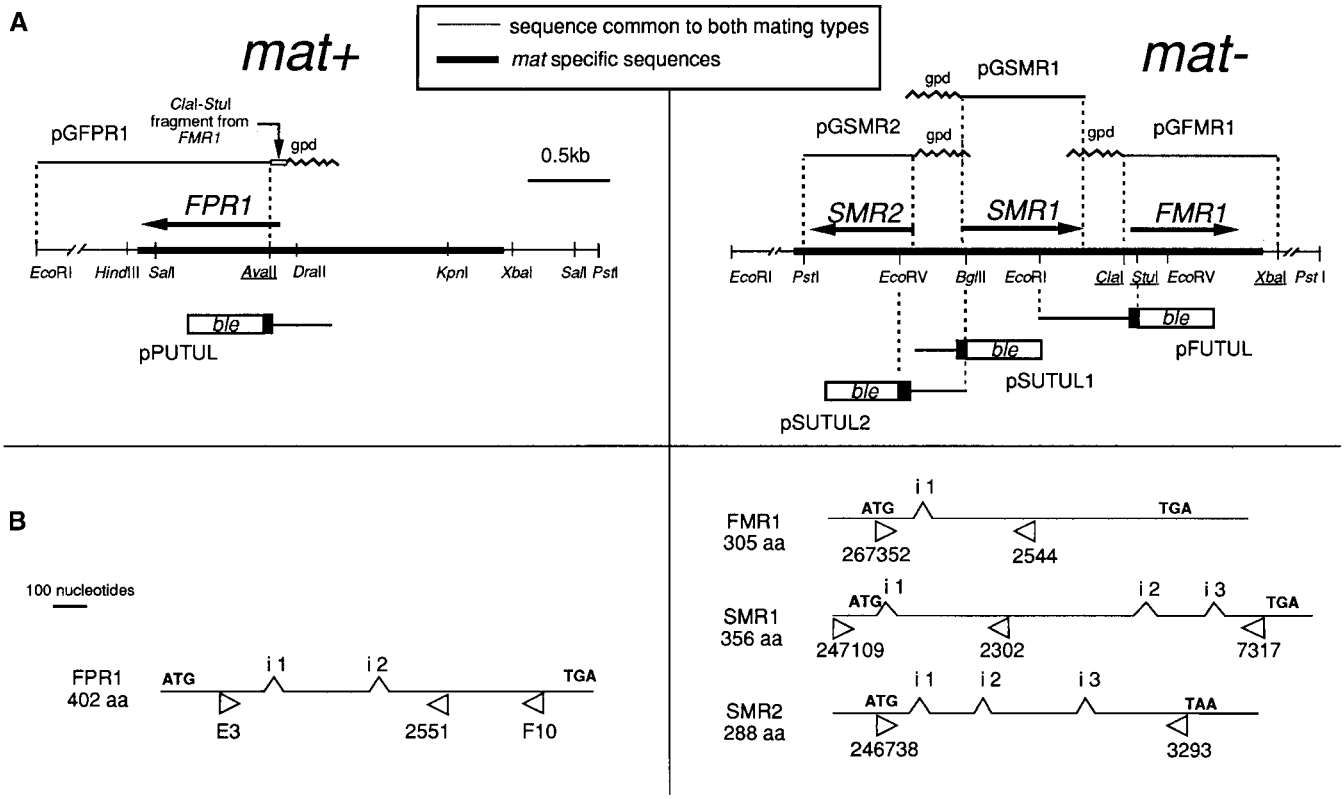

Figure 1.—Functional structure of the *mat*+ and *mat*− mating types. (A) Restriction map of the *mat* locus (on chromosome *I*, the centromere-proximal end is left of this map). The underlined sites for restriction enzymes are not unique. Arrows indicate position and orientation of coding sequences. The *mat*-specific sequences fused to the *ble* reporter gene and to the *gpd* promoter and the name of the relevant plasmids are shown below and above the map, respectively. (B) Detailed structure of the genes with position of their introns (i 1, i 2, i 3). Arrowheads below the map indicate the position of the oligonucleotides used in RT-PCR experiments and/or PCR analysis of the integrated transgene in transformants.

formants were recovered at a higher frequency with pPUTUL (75%) and pSUTUL2 (83%) than with pFUTUL (37%). Primary transformants were expected to manifest a heterogeneity of basic resistance to phleomycin. First, they contained variable proportions of transformed and untransformed nuclei because protoplasts are often plurinucleate. Second, transforming DNA integrates at random chromosomal locations that may have different *cis*-effects on the transcription activity of the *ble* gene. Third, inactivation of an active transgene during integration of the transforming DNA is a frequent event. The potential heterogeneity was examined by testing 30–100 primary (leu$^+$) transformants obtained with each fusion for their resistance to phleomycin, and the MIC was determined for two or three purified transformants (Table 1). Purified transformants carrying the *FPR1::ble* fusion (pPUTUL) and *SMR2::ble* fusion (pSUTUL2) could still grow at 100 μg/ml phleomycin while transformants carrying the *FMR1::ble* fusion were inhibited at 30 μg/ml. These data suggest that the expression level of the *FPR1::ble* and *SMR2::ble* fusions is higher than the expression level of the *FMR1::ble* fusion. In contrast, all transformants carrying *SMR1::ble* fusion (pSUTUL1) were as sensitive to phleomycin as the wild-type strain. PCR assays (see materials and methods) and Southern analysis of DNA from three purified strains transformed with pSUTUL1 were carried out and confirmed that the *5′SMR1::ble* transgenes were not rearranged (data not shown).

Vegetative repression of *SMR2* transcription requires an upstream *cis* element: The two above-cited methods gave conflicting data with respect to *SMR2*, since *SMR2* mRNA were not detected in mycelium by RT-PCR analysis whereas the *5′SMR2::ble* fusion was found to be expressed vegetatively. These data suggest that the vegetative transcription of the *5′SMR2::ble* fusion results from the loss of a regulatory *cis* element involved in transcriptional repression or in transcript instability during the vegetative phase. RT-PCR assays were performed on RNA prepared from mycelia of *mat*+ and *mat*− transformants bearing different transgenic constructs containing *SMR2*. *SMR2* cDNA was not detected in *mat*+ transformants bearing the *SMR2* coding sequence and 4.7 kb upstream of the *SMR2* translation initiation covering the entire *mat*− region at an ectopic position (see Figure 1A). In contrast, *SMR2* mRNA were detected in mycelium of *mat*+ and *mat*− strains that contain the

TABLE 1

Resistance level to phleomycin displayed by the transformants carrying the 5′ *mat::ble* gene fusion

Transforming vector[a]		Frequency of phleo^R transformants	MIC[b] (μg/ml)
	Wild type		10–20
PUT703	gpd — ble[c]	Not tested	>100
pFLUT	unknown — 13aa — ble	1/60	10–20
pPUTUL	5′ FPR1 — 1000 bp — 10aa — ble	68/90	>100
pFUTUL	5′ FMR1 — 1000 bp — 13aa — ble	22/60	~30
pSUTUL1	5′ SMR1 — 590 bp — 4aa — ble	0/30	10–20
pSUTUL2	5′ SMR2 — 740 bp — 22aa — ble	24/29	>100

[a] For each construct the size of the 5′ UTR (in base pairs) and the size of the coding sequence (in amino acids) belonging to the *mat* gene are indicated. Localization of those sequences in the physical map is shown in Figure 1A. In pFLUT and pFUTUL the fusion gene contains the sequence coding the first 13 amino acids of FMR1.

[b] The MIC of phleomycin was determined on two or three purified transformants harboring each fusion (see materials and methods). When a high frequency of transformants resistant to phleomycin was obtained, only resistant transformants were purified.

[c] The *ble* gene is driven by the *A. nidulans gpd* promoter.

SMR2 coding sequence and 1.4 kb upstream of the *SMR2* first codon. These data suggest that an element required for the repression of *SMR2* transcription is present in the region between 1.4 kb and 4.7 kb upstream of the *SMR2* translation start.

Deregulated *mat* genes complement *mat* mutants and do not alter vegetative phenotype: To promote expression or overexpression of *mat* genes during vegetative growth, we constructed gene fusions between the glycero-phosphate-3-dehydrogenase (*gpd*) promoter of *A. nidulans* and the coding region of the four *mat* genes, including the initiation codon. Constructs were cloned in the pUL plasmid carrying the *leu1* selective marker, giving rise to pGFPR1, pGFMR1, pGSMR1, and pGSMR2 plasmids (Figure 1A). Each plasmid was introduced into a strain suitable for examining the functions of the *gpd::mat* fusion, generally the Δ*mat* mutant deleted for *mat* information (Coppin *et al.* 1993). When necessary additive wild-type *mat* genes were introduced simultaneously to the *gpd::mat* fusion. Between 10 and 20 (leu$^+$) primary transformants carrying each plasmid were crossed with testers to determine their sexual phenotype. The phenotypic assays described in Table 2 indicate whether the *gpd::mat* fusion is functional but do not give information on its expression level. Transformants with the expected sexual phenotype were recovered with each of the four constructs. The data were as follows:

1. Transformants carrying *gpd::FPR1* displayed full mat+ activity (fertilization of a *mat*− partner giving rise to fertile perithecia producing asci).
2. Transformants carrying *gpd::FMR1* displayed partial mat− activity (fertilization of a *mat*+ partner giving rise to poorly fertile perithecia). The postfertilization function was examined by introducing simultaneously into the Δ*mat* strain pGFMR1 and pSUT12 containing the two other *mat*− genes (*SMR1* and *SMR2)* with the *ble* selective marker. The cotransformants displayed a wild-type *mat*− activity (fertilization of a *mat*+ partner giving rise to fertile perithecia producing asci).
3. The cotransformants containing both the *gpd::SMR1* fusion and a *mat*+ transgenic information were crossed with the *mat*− (*SMR1::ura5*) mutant, disrupted within *SMR1* at the resident *mat*− locus. The cross yielded abundant progeny, whereas a cross of the *mat*− (*SMR1::ura5*) mutant with the *mat*+ wild-type strain was sterile. Internuclear complementation, previously demonstrated with the native *SMR1* gene (Arnaise *et al.* 1997), was thus also observed for the *gpd::SMR1* fusion.
4. The pGSMR2 plasmid was introduced into a *leu1-1 mat*− (*SMR2::ura5*) recipient since only intranuclear complementation was observed for the native *SMR2* gene (Arnaise *et al.* 1997). The *mat*− (*SMR2::ura5*) strain provided only uniparental progeny in crosses with a *mat*+ tester (Arnaise *et al.* 1997), whereas the transformants containing the *gpd::SMR2* fusion also gave biparental progeny, thus indicating efficient complementation of the mutant phenotype.

The data demonstrate that the four *mat* genes are active when driven by the foreign *gpd* promoter. Moreover, during these tests, no *gpd::mat* fusion was found to produce an effect on viability, growth, or morphology of the recipient strain.

The functional assays allowed us to determine whether the *gpd::mat* fusions were active during sexual reproduction but did not allow us to determine whether they were expressed vegetatively. In particular, replacement of the native promoter of *SMR1* by the *gpd* promoter was expected to induce its vegetative expression. To check the presence of *gpd::SMR1* mRNA, RT-PCR assays were performed on RNA prepared from mycelia of one transformant bearing a functional *gpd::SMR1* fusion. A product of the expected size was detected using a primer localized close to the stop codon in association with a *gpd* specific primer, indicating that the *gpd::SMR1* fusion was vegetatively transcribed.

TABLE 2

Functional tests performed to determine expression of the *mat* genes under control of the *A. nidulans gpd* promoter

Recipient strains	Transforming plasmids	Transforming phenotype	Sexual phenotype[a]	Transformants displaying sexual phenotype/total
leu1-1 Δ*mat*	pGFPR1	leu$^+$	mat+, spo+	9/20[b]
leu1-1 Δ*mat*	pGFMR1	leu$^+$	mat−, spo−	7/20
leu1-1 Δ*mat*	pGFMR1 and pSUT12 (*SMR1 SMR2*)	leu$^+$, phleo^R	mat−, spo+	6/15
leu1-1 Δ*mat*	pGSMR1 and pucES+ (*mat*+)	leu$^+$, mat+	spo+ in cross with *SMR1::ura5*	7/15[b]
leu1-1 mat− *(SMR2::ura5)*	pGSMR2	leu$^+$	mat−, spo+	5/11

[a] Expression of the *gpd::mat* genes can be tested by determining the fertilization ability (mat+ or mat−) on a tester of opposite mating type and/or the production of a progeny (spo+, abundant progeny with Mendelian segregation of the parental genetic markers; spo−, no progeny.

[b] Integrity of the transgenic fusion gene was checked by PCR analysis in one or two transformants.

The combination of deregulated *mat* genes produces a lethal phenotype: With the help of the transgenic strains obtained by transformation with the different *mat* constructs, we performed numerous crosses to obtain different combinations of the deregulated *mat* genes. In the course of the genetic analysis of some crosses, we observed a high frequency of ascospores unable to germinate. Tetrad analysis allowed us to determine their genotype: the lethal ascospore carried the *mat*+ resident mating type (*FPR1*) and both *gpd::FMR1* and *SMR2* transgenes, that is to say an artificial association of the three IR genes. To further investigate this phenomenon, new transformants more suitable for genetic analysis were constructed. For that purpose, the *gpd::FMR1* fusion was cloned in a plasmid carrying the *ble* gene as selective marker (pPgFMR1). A plasmid containing the native *FMR1* gene with its own 5′ UTR (pPaFMR1) was also constructed to examine the role of the promoter. A *SMR2* gene with a 1.4-kb 5′ UTR was cloned in a plasmid carrying the *hph* gene, conferring resistance to hygromycin (pCBSMR2). The segregation of *FMR1* and *SMR2* transgenes could thus be easily followed through resistance to phleomycin (phleoR) and hygromycin (hygroR), respectively.

Transformants (hygroR) were recovered upon transformation of the *mat*+ recipient with the pCBSMR2 plasmid. Introduction of the *SMR2* transgene into a *mat*+ strain was previously found to induce the enlargement of female organs that do not develop if they are not fertilized (S. Arnaise, personal communication). This phenotype was used to determine whether the (hygroR) transformants carried a functional *SMR2*. Such transformants were genetically purified by crossing with a *mat*− wild-type strain, and homokaryotic *mat*+ and *mat*− progeny harboring the *SMR2* and *hph* transgenes were recovered. Three homokaryotic transformants were generated from three independent primary transformants in which the plasmid had integrated at different chromosomal locations. They are named *SMR2-4*, *SMR2-16*, and *SMR2-19*: in the nomenclature *SMR2-x*, *x* specifies the integration locus of the *SMR2* transgene. The same nomenclature is used to design each *mat* gene introduced by integrative transformation. Transformants carrying the pCBSMR2 construct must express *SMR2* in hyphae because the gene does not contain the negative *cis* element that represses its transcription in mycelium. This prediction is confirmed by the detection of a fragment with the expected size for cDNA in RT-PCR assays performed on RNA extracts from the *mat*+ *SMR2-19* strain (data not shown). *SMR2^c* will subsequently designate this constitutive *SMR2* transgene.

In the same way, (phleoR) *mat*+ primary transformants obtained with the pPgFMR1 or the pPaFMR1 plasmids were used to generate homokaryotic *mat*+ and *mat*− progeny carrying the *gpd::FMR1* fusion (*GFMR1-1*, *GFMR1-2*) or the *FMR1* transgene (*FMR1-1*, *FMR1-3*, *FMR1-7*). Introduction of these plasmids into a *mat*+ recipient induced self-fertilization and ability to fertilize a *mat*+ tester provided the *FMR1* transgene was active. Only primary transformants exhibiting this phenotype were selected. QC-RT-PCR detection of *FMR1* transcripts in total RNA extracted from *GFMR1-1*, *FMR1-1*, and wild-type *mat*− strains indicated that transcription of *FMR1* is at least 10 times higher in *GFMR1-1* strain than in *FMR1-1* and wild-type strains (materials and methods). The *GFMR1-5* transformant was previously obtained with the pGFMR1 plasmid carrying the *gpd::FMR1* fusion associated with the *leu1* gene.

Crosses were performed between *mat*+ strains carrying a *SMR2^c* transgene and *mat*− strains carrying a *gpd::FMR1* fusion and submitted to genetic analysis. Data are presented in Table 3 and in Table 4 (first four columns). The most important finding is that no viable *mat*+ *SMR2^c GFMR1* homokaryotic progeny was recovered, while the seven other possible genotypes were obtained at equivalent frequency (except in crosses with *SMR2-16* because the transgene is linked to *mat*+). Tetrad analysis (materials and methods) indicated that

TABLE 3

Homokaryotic progeny obtained in crosses of *mat*+ strains carrying the (*SMR2, hph*) transgenes (*SMR2-19, SMR2-4, SMR2-16*) with the *mat*− strain carrying the (*gpd::FMR1, leu1*) transgene (*GFMR1-5*)

Phenotype				*GFMR1-5* × *SMR2-19*	*GFMR1-5* × *SMR2-4*	*GFMR1-5* × *SMR2-16*[a]
hyg	mat	self	Inferred genotype			
S	mat+	—	*mat*+	3	4	0
S	mat+	+	*mat*+ *GFMR1*	7	7	0
R	mat+	+	*mat*+ *SMR2*[c][b]	5	5	10
R	mat+	+	*mat*+ *SMR2*[c] *GFMR1*	0	0	0
S	mat−	—	*mat*−	14	9	14
S	mat−	—	*mat*− *GFMR1*			
R	mat−	—	*mat*− *SMR2*[c]	2	6	0
R	mat−	—	*mat*− *SMR2*[c] *GFMR1*[c]	2	2	0

Abbreviations are as follows: hyg, hygromycin; R and S, resistant and sensitive; mat, resident mating type; self, formation of perithecia or microperithecia on the homokaryotic mycelium.

[a] (*SMR2-16*, *hph*) are genetically linked to *mat*+.

[b] Strains unable to fertilize a *mat*+ strain, a function expected if *GFMR1-5* were present.

[c] Unpigmented, female sterile mycelia.

the absent genotype was attributable to immature ascospores that had not germinated. This genetic association of *mat* genes was thus responsible for an autonomous ascospore lethal phenotype, that is, a phenotype controlled by the nuclei within the ascospore itself. Abundant asci with morphologically normal ascospores were produced in all crosses, indicating that proper sexual development occurred before ascospore delimitation. Heterokaryotic ascospores interpreted as containing both *mat*+ *SMR2*[c] *GFMR1* and *mat*− wild-type nuclei were unable to germinate, which indicated that the lethality was dominant. Contrary to the *mat*+ *SMR2*[c] *GFMR1* ascospores, the *mat*− *SMR2*[c] *GFMR1* ascospores were viable. Nevertheless, on growth medium they gave rise to an unpigmented and flat mycelium that grew as well as the wild-type strain but failed to form aerial hyphae and rarely differentiated female organs (at least 200 times less than the wild type). Consequently, when used as female parent in a cross, only 10 to 100 fruiting bodies were produced on a petri dish in contrast to the thousands produced by a wild-type cross. The six other genotypes listed in Tables 3 and 4 did not confer any particular phenotype. Finally, a *mat*− *SMR2-19 GFMR1-2* strain issued from this analysis was crossed with a *mat*+ strain. As previously, *mat*+ *SMR2-19 GFMR1-2* progeny were not obtained. The data indicated that the lethality phenomenon occurs whatever the initial association of the *SMR2* and *GFMR1* transgenes in the parental strains (one transgene in each parent as in Table 3 and Table 4 or both in the same parent).

Crosses were also performed between *mat*+ *SMR2*[c] strains and *mat*− *FMR1* strains (Table 4, last six columns). By contrast to crosses with *mat*− *GFMR1* strains, mature *mat*+ *SMR2*[c] *FMR1* ascospores giving rise to viable mycelium were recovered. Although the *SMR2-19 FMR1-3* and *SMR2-19 FMR1-7* associations did not confer an ascospore lethal phenotype in a *mat*+ strain, they nonetheless impaired the growth rate and morphology of the mycelium, which was devoid of aerial hyphae. In a *mat*− strain, they conferred the phenotypic alteration (unpigmented female sterile mycelium) already observed in *mat*− *SMR2*[c] *GFMR1* strains (Table 5). By contrast, *SMR2*[c] *FMR1-1* associations did not impair the phenotype, whatever might be the mating-type resident haplotype (Table 5). One may assume that the vegetative effects are less drastic because the *FMR1* transgenes are less expressed than are the *gpd::FMR1* fusions as indicated by QC-RT-PCR experiments. Moreover, the integration site of the ectopic *FMR1* copy may influence its expression level, which could be lower in *FMR1-1* than in *FMR1-3* and *FMR1-7* transformants. The same rationale can be applied to *SMR2* to explain the phenotypic differences between *mat*+ *FMR1-3 SMR2-4* and *mat*+ *FMR1-3 SMR2-19* strains (Table 5).

In conclusion, the association of a *FPR1* (*mat*+) resident gene with a vegetatively expressed *SMR2* transgene and a *gpd::FMR1* transgene appears to be lethal. Replacement of the *gpd::FMR1* by the *FMR1* transgene results in viable strains that nevertheless exhibited impaired growth rate and mycelium morphology. Vegetative expression of *SMR2* was confirmed by detection of a fragment with the expected size for cDNA in RT-PCR assays performed on RNA extracts from the *mat*− *SMR2-19 GFMR1-2* strains (data not shown).

To examine the role of the resident *FPR1* gene, the *SMR2* and *gpd::FMR1* transgenes were introduced into a Δ*mat* strain by crossing the *mat*− *SMR2-19 GFMR1-1* strain with a Δ*mat* strain carrying the 6.3-kb *Eco*RI-*Sal*I fragment from the *mat*+ locus (see Figure 1A). Progeny with the Δ*mat SMR2-19 GFMR1-1* genotype were recovered that displayed a normally pigmented mycelium that grew well. Viability of the Δ*mat SMR2-19 GFMR1-1*

TABLE 4

Homokaryotic progeny obtained in crosses of strains carrying the (*SMR2, hph*) transgenes (*SMR2-19*, *SMR2-4*, *SMR2-16*) with strains carrying the (*gpd::FMR1, ble*) or (*FMR1, ble*) transgenes (*GFMR1-1*, *GFMR1-2* and *FMR1-1*, *FMR1-3*, *FMR1-7*)

Phenotype					*GFMR1-1* × *SMR2-19*	*GFMR1-1* × *SMR2-4*	*GFMR1-2* × *SMR2-19*	*GFMR1-2* × *SMR2-16*[a]	*FMR1-1* × *SMR2-19*	*FMR1-1* × *SMR2-4*	*FMR1-1* × *SMR2-16*[a]	*FMR1-3* × *SMR2-19*	*FMR1-3* × *SMR2-4*[b]	*FMR1-7* × *SMR2-19*
hyg	phl	mat	self	Inferred genotype										
S	S	mat+	—	*mat+*	7	6	3	0	5	5	0	3	0	4
S	R	mat+	+	*mat+ FMR1*	5	9	8	0	1	3	0	1	5	2
R	S	mat+	+	*mat+ SMR2*[c]	4	7	3	7	1	2	7	2	7	2
R	R	mat+	+	*mat+ FMR1* SMR2[c]	0	0	0	0	5	4	4	1	1	2
S	S	mat−	—	*mat−*	3	1	4	7	5	5	7	1	1	4
S	R	mat−	—	*mat− FMR1*	2	6	3	8	4	2	4	1	5	4
R	S	mat−	—	*mat− SMR2*[c]	6	8	4	0	0	2	0	1	9	3
R	R	mat−	—	*mat− FMR1 SMR2*[c]	3	5	1	0	5	3	0	2	0	3

Abbreviations are as in Table 3. Phl, phleomycin.

[a] (*SMR2-16*, *hph*) are genetically linked to *mat+*.

[b] (*SMR2-4*, *hph*) are genetically linked to (*FMR1-3*, *ble*).

ascospores definitely confirms that the deregulated *mat−* genes must be associated with the resident *FPR1* (*mat+*) gene to confer the ascospore lethal phenotype.

Observations made in the course of our study show that *P. anserina* transgenic strains exhibit some instability. In particular, sectors with increased growth rate appeared frequently from the poorly growing *mat+ SMR2-19 FMR1-3* or *mat+ SMR2-19 FMR1-7* mycelium. This phenomenon was investigated by crossing several sectors with the *mat−* wild-type strain. Although resistance to hygromycin (*SMR2*, *hph*) and phleomycin (*FMR1*, *ble*) segregated normally in the offspring, the sexual phenotype associated with either *SMR2* or *FMR1* transgene was lost. PCR reactions were performed on DNA extracted from sectors and from some of their progeny with a pair of primers specific to the inactive transgene: no band was detected, whereas the specific band was present in assays with DNA from the original mycelium (data not shown). The growth improvement in the sectors is thus caused by the loss of one of the transgenes without concomitant loss of the associated resistance marker. Consequently, to avoid any misinterpretation of a phenotype, the presence of a transgene was never deduced solely from the phleR or hygroR phenotype, but also using a functional test when possible and/or a PCR assay when necessary. To date, excision of an integrated plasmid or transgene in *P. anserina* transformants was considered a rare event, perhaps mistakenly. The situation we report is particularly well adapted to reveal such an event since the transgenes are "toxic." First, there is a selection pressure in favor of nuclei that have lost one transgene. Second, the loss of a transgene is associated with an increased growth rate and aerial hyphae production and thus with a directly observable phenotypic change.

Integration of a *gpd::SMR1* fusion restores viability to the *mat+ SMR2-19 GFMR1-2* strain: *FPR1*, *FMR1*, and *SMR2* control a sexual development-specific function, internuclear recognition. As explained in the Introduction, the fourth *mat* gene, *SMR1* (*mat−*), is assumed to act downstream of *FPR1*, *FMR1*, and *SMR2*. We therefore tested the effect of the *gpd::SMR1* fusion on the phenotype resulting from the expression of *FPR1*, *FMR1*, and *SMR2* in the same nucleus. The *gpd::SMR1* transcriptional fusion was used to force expression of *SMR1* in vegetative mycelium since transcription of *SMR1* was observed only in perithecia (see above). The *mat− SMR2-19 GFMR1-1* and *mat− SMR2-19 GFMR1-2* strains were crossed with *mat+* strains harboring the *gpd::SMR1* fusion (*GSMR1-4* and *GSMR1-5*). These were obtained in two steps. First, the *mat+ leu1-1* strain was transformed with the pGSMR1 plasmid and (leu^+) primary transformants were recovered. Second, the transformants were crossed with the *mat−* (*SMR1::ura5*) strain. The sterility of this strain was complemented by the *gpd::SMR1* transgene carried by the mating partner, allowing these crosses to produce progeny among which we identified the expected *mat+ gpd::SMR1* genotype.

TABLE 5

Phenotypes of *mat+ FMR1 SMR2* and *mat− FMR1 SMR2* strains

	Phenotype				
	mat+ context		*mat−* context		
Genotype	Ascospore germination	Growth	Growth	Mycelial pigmentation	Female fertility[a]
WT	+	+	+	+	+
GFMR1 SMR2[c]	—	—	+	—	—
FMR1-1 SMR2-19	+	+	+	+	+
FMR1-1 SMR2-4	+	+	+	+	+
FMR1-1 SMR2-16	+	+		Not determined	
FMR1-3 SMR2-19	+	Poor	+	—	—
FMR1-3 SMR2-4	+	+		Not determined	
FMR1-7 SMR2-19	+	Poor	+	—	—

[a] +, protoperithecia are formed; —, no (or few) protoperithecia are formed.

Data of the crosses *mat− SMR2-19 GFMR1-1/GFMR1-2* × *mat+ GSMR1-4/GSMR1-5* are presented in Table 6. The segregation of the *gpd::SMR1* in *mat+* progeny was scored by ability to restore fertility in sexual cross with the *mat−* (*SMR1::ura5*) mutant. Since no simple functional test for *gpd::SMR1* in *mat−* progeny was possible, the presence of the *gpd::SMR1* transgene was established by PCR analysis when necessary. If the *gpd::SMR1* fusion gene acts as a suppressor of ascospore lethality, (mat+ hygroR phleoR) mycelium corresponding to the *mat+ SMR2-19 GFMR1 GSMR1* genotype should be recovered at a 6.25% frequency of the homokaryotic ascospores (16 genotypes at equivalent frequency were expected in a cross involving four unlinked genetic markers). Progeny with this phenotype were not obtained in crosses with *mat+ SMR2-19 GFMR1-1* (two final columns of Table 6). In contrast, in crosses of *mat+ SMR2-19 GFMR1-2* with *mat+ GSMR1-4* and *mat+ GSMR1-5* (first two columns of Table 6), 8.4% and 6.7%, respectively, of viable homokaryotic ascospores generating mycelium with the expected phenotype were found. The presence of the *gpd::SMR1*, *SMR2*, and *GFMR1* transgenes de-

TABLE 6

Homokaryotic progeny obtained in crosses of strains carrying the (*SMR2, hph*) and (*gpd::FMR1, ble*) transgenes (*SMR2-19 GFMR1-1* and *SMR2-19 GFMR1-2*) with strains carrying the (*gpd::SMR1, leu1*) transgenes (*GSMR1-4* and *GSMR1-5*)

Genotype[a]	*GSMR1-4* × *SMR2-19 GFMR1-2*	*GSMR1-5* × *SMR2-19 GFMR1-2*	*GSMR1-4* × *SMR2-19 GFMR1-1*	*GSMR1-5* × *SMR2-19 GFMR1-1*
mat+	2	3	1	2
mat+ GSMR1	1	2		
mat+ GFMR1	3	7	6	7
mat+ GFMR1 GSMR1	3	9		
mat+ SMR2[c]	0	6	8	8
mat+ SMR2[c] *GSMR1*	3	6		
mat+ SMR2[c] *GFMR1*	0	0	0+ (0/56)[c]	0
mat+ SMR2[c] *GFMR1 GSMR1*	1 + (10/104)[b]	6		
mat− ± GSMR1	5	10	2	4
mat− GFMR1 ± GSMR1	3	16	7	5
mat− SMR2[c] *± GSMR1*	3	14	4	2
mat− SMR2[c] *GFMR1 ± GSMR1*	3 + (11/104)[b]	11	0 + (7/56)[c]	1

Abbreviations are as in Table 5.

[a] Genotypes were deduced from the phenotypic tests presented in Table 4; segregation of *gpd::SMR1* in the *mat+* progeny was determined by crossing with *mat−* (*SMR1::ura5*) only when (mat+ phleR hygR) progeny were obtained (two first columns). In the two last columns each number is for two genotypes ± *GSMR1*.

[b] An additive cross was performed and 104 homokaryotic progeny analyzed. Ten (mat+ hygR phlR) progeny were obtained and were found to carry the *gpd::SMR1* transgene.

[c] An additive cross was performed and 56 homokaryotic progeny analyzed. No (mat+ phleR hygR) progeny were obtained.

duced from functional tests was also confirmed by genomic DNA PCR analysis in some progeny. Although the *mat+ SMR2-19 GFMR1-2 GSMR1* ascospores germinated well, the colonies displayed a slight morphological alteration on the germination medium as compared to wild type (smaller thallus with a less regular margin). Moreover, after transfer on minimal medium, mycelial growth is very slow and irregular. The (mat− hygroR phleoR) progeny were submitted to PCR analysis to test the presence of the *gpd::SMR1* transgene. In fact these progeny corresponded to two genotypes, *mat− SMR2-19 GFMR1-2* and *mat− SMR2-19 GFMR1-2 GSMR1*, which gave the same altered phenotype: unpigmented female sterile mycelia with no aerial hyphae. These genetic data indicated that *SMR1* did not suppress the mutant phenotype conferred by the *mat− SMR2-19 GFMR1-2* association.

Finally, to confirm the suppression of lethality by *SMR1*, a *mat− SMR2-19 GFMR1-2 GSMR1-5* progeny was crossed with a *mat+ GSMR1-5* strain. The *gpd::SMR1* transgene was present in both parents, and only viable ascospores were recovered. Among the 80 homokaryotic ascospores analyzed, 11% were (mat+ hygroR phleoR) and 9% were (mat− hygroR phleoR), in agreement with the 12.5% expected for each class.

DISCUSSION

We have studied the means by which mating-type genes are regulated during the life cycle of *P. anserina* to choose a strategy to deregulate them. We have further examined the physiological consequences of forcing their expression in vegetative hyphae.

RT-PCR analyses and fusions of mating-type genes with reporter genes revealed that during the vegetative phase *FMR1* and *FPR1* are expressed whereas *SMR1* and *SMR2* are transcriptionally silent. The vegetative expression of *FMR1* and *FPR1* genes is in agreement with their role in fertilization (Debuchy and Coppin 1992) and with the observation that hyphae can occasionally substitute to microconidia for fertilization. Mature transcripts of the four mating-type genes have been detected in the fertilized female organs, suggesting that some unknown factors control the transcription of *SMR1* and *SMR2* in the perithecium. A negative *cis*-acting element has been localized in a region 1.4 kb to 4.7 kb upstream of *SMR2*. Preliminary experiments suggest that the *SMR2* silencer is present in the *FMR1* sequence, 2.4 kb upstream of *SMR2* translation start.

A comparison of the transcription pattern of *Neurospora crassa* (Ferreira *et al.* 1996) and *P. anserina* mating-type genes indicates that these genes are regulated at different steps of their expression in the the two fungi. In *N. crassa*, *mt A-1* (similar to *FMR1*), *mt A-2* (similar to *SMR1*), and *mt A-3* (similar to *SMR2*) are transcribed in mycelium on solid vegetative medium and crossing medium, but it is not known if they are expressed in vegetative cells or only in reproductive structures. The authors proposed that translation of *mt A-2* and *mt A-3* messengers may be developmentally regulated through small open reading frames that are present downstream of the 5′ end in these messengers.

Since transcriptional regulation is the primary regulatory control of expression of the *P. anserina mat* genes, we deregulated them by replacing their natural promoter with the *A. nidulans gpd* promoter. Each of the four *gpd::mat* fusions was found to be functional since it can complement a null or a mutated allele. No effect was noticed when one fusion was introduced in a *mat+* or *mat−* wild-type strain. Crosses were performed between transgenic strains to associate the three IR genes in the same nucleus. We were unable to construct *mat+* (*FPR1*) strains containing a constitutively transcribed $SMR2^c$ gene and a *gpd::FMR1* fusion (Table 5). Ascospores with this genotype were nevertheless recovered but they were unable to germinate, which indicated that this genetic association was lethal. By contrast, homokaryotic *mat+* (*FPR1*) strains containing a constitutively transcribed $SMR2^c$ transgene and a *FMR1* transgene driven by its own promoter were obtained (Table 5). They exhibited a flat mycelium with reduced growth. The *gpd::FMR1* fusion was found to be at least 10 times more transcribed than *FMR1* driven by its own promoter, suggesting that the overexpression of *FMR1* in a $SMR2^c$ *mat+* strain is lethal, while a lower expression of *FMR1* has a less drastic effect. Coexpression of the three IR genes thus results in a partial or complete inhibition of growth. We propose that this phenomenon mimics the events that occur during sexual reproduction. A cross between a *mat+* strain and the *mat−* (*SMR1::ura5*) mutant, which contains functional *FMR1* and *SMR2* genes, displays a phenotype in agreement with this interpretation. This cross is sterile, although fertilization has occurred. Cytological observations indicate that development of perithecia is blocked before formation of ascogenous hyphae (Arnaise *et al.* 1997). Since IR genes are functional, it is likely that IR occurs normally in this cross and that development arrests shortly after the recognition stage. We postulate that this developmental arrest is a programmed event similar to the growth arrest observed in the mycelium as a result of the expression of IR genes. In the fruiting body, this arrest may be required for the synchronization of *mat+* and *mat−* nuclei before entry into the ascogenous hyphae, where they undergo simultaneous mitoses (Simonet and Zickler 1972). Construction of a *mat+* (*FPR1*) strain containing a $SMR2^c$ and a *gpd::FMR1* transgene was found to be possible by simultaneously introducing the *gpd::SMR1* fusion. The suppression of vegetative growth inhibition resulting from vegetative coexpression of *FPR1*, *FMR1*, and *SMR2* by a *gpd::SMR1* fusion confirms that *SMR1* operates downstream of IR genes. This is in agreement with the hypothesis of Arnaise *et al.* (1997), who proposed that during sexual development

SMR1 is required after IR for the initial development of biparental ascogenous hyphae. In the fruiting bodies, *SMR1* function would thus be required to remove the developmental inhibition triggered by IR.

The hypothesis that recently proposed that IR is mediated by a pheromone response pathway (Debuchy 1999) may help us to understand what happens at the molecular level when IR genes are expressed. The possible involvement of a pheromone cascade in IR suggests that the accompanying growth arrest may be similar to the G1 cell-cycle arrest observed in *Saccharomyces cerevisiae* in response to the activation of the pheromone response pathway (reviewed in Cross *et al.* 1988). In yeast, constitutive activation of this pathway caused by disruption of the structural gene for the Gα protein results in a haploid-specific lethal phenotype due to cell-cycle arrest (Miyajima *et al.* 1987). Similarly, in *P. anserina*, heterochronic vegetative coexpression of the three IR genes in the same nucleus could generate a constitutive activation of the pheromone signal transduction pathway and thus provoke nuclear arrest in G1.

The association of the *SMR2^c* and the *gpd::FMR1* transgenes, lethal in the *mat*+ strain, is viable in the *mat*− strains but leads to phenotypic alterations. The mycelium of all *mat*− *SMR2^c GFMR1* and some *mat*− *SMR2^c FMR1* strains is unpigmented, devoid of aerial hyphae, and almost totally female sterile (Table 5). However, these strains grow as well as the wild-type strain in contrast to the *mat*+ *SMR2^c FMR1* strains. The finding that the Δ*mat SMR2-19 GFMR1-1* strain has a wild-type phenotype in contrast to the *mat*− *SMR2-19 GFMR1-1* strain that displayed a mutant phenotype demonstrates that the resident *mat*− information is important in determining the morphological alterations. It has been established that differentiation of sexual reproductive structures is not controlled by the mating-type genes, since it occurs in the Δ*mat* mutants (Coppin *et al.* 1993). Consequently, female sterility caused by forced vegetative expression of *SMR2* and *FMR1* in the *mat*− context (and the other phenotypic alterations) may be an indirect effect resulting from deregulation of the expression of the *mat*− genes. This deregulation might alter fungal physiology and, more particularly, generate a bypass of the female differentiation pathway. In accord with this hypothesis, the pleiotropic phenotype of the *mat*− *SMR2^c GFMR1* strains is not suppressed by the *gpd::SMR1* fusion, contrary to the lethal phenotype resulting from association of the three IR genes.

All available lines of evidence suggest that vegetative expression of mating-type genes results in the activation of their target genes and mimics the transitory events occurring within the fruiting body. Ectopic expression of the mating-type genes is a valuable tool for the comprehension of fruiting-body development. The characterization of vegetative growth inhibition triggered by coexpression of the three IR genes opens a new field of investigation. This phenotype provides a powerful genetic screen for selecting suppressors and identifying possible target genes of the transcription factors encoded by the mating-type genes. Moreover, in nonlethal associations leading to a reduced mycelial growth rate, we have available biological material for the identification of specific mRNA by differential hybridization with RNA from a wild-type strain.

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