The mod-A Suppressor of Nonallelic Heterokaryon Incompatibility in Podospora anserina Encodes a Proline-Rich Polypeptide Involved in Female Organ Formation

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ABSTRACT

Vegetative incompatibility in fungi results from the control of heterokaryon formation by the genes present at het loci. Coexpression of antagonistic het genes in the same hyphae leads to a lethal process. In Podospora anserina, self-incompatible strains containing nonallelic incompatible genes in the same nucleus are inviable as the result of a growth arrest and a lytic process. Mutations in suppressor genes (mod genes) can restore the viability. These mod mutations also interfere with developmental processes, which suggests common steps between the incompatibility reaction and cellular differentiation. The mod-A locus, responsible for growth arrest in the self-incompatible strains, is also involved in the control of the development of female organs. The mod-A gene was isolated. An open reading frame 687 amino acids long was identified. The MOD-A-encoded polypeptide is rich in proline residues, which are clustered in a domain containing a motif that displays similarity to SH3-binding motifs, which are known to be involved in protein-protein interactions. Construction of a strain deleted for mod-A confirmed that the product of this gene involved in differentiation is a key regulator of growth arrest associated with vegetative incompatibility.

In filamentous fungi, fusion between genetically distinct individuals is restricted as the result of somatic or vegetative incompatibility. Vegetative incompatibility is widespread in these organisms and is the consequence of the incapacity to form viable heterokaryotic cells following anastomosis between filaments of unlike geno-type. The phenomenon is controlled by differences at specific loci named het loci, which have been genetically characterized in some filamentous fungi such as Neurospora crassa, Podospora anserina, Aspergillus nidulans, and Cryphonectria parasitica (for reviews see Glass and Kulda 1992; Begueret et al. 1994). In P. anserina, the antagonist het genes may be alternate alleles at a single locus or nonallelic genes from independent loci (Bernet 1967). In the latter case, different alleles can be observed at each nonallelic het locus. The formation of heterokaryons leads to compatible or incompatible genetic combinations of het genes. Heterokaryotic cells resulting from an incompatible combination of allelic genes (allelic incompatible interaction) or of nonallelic het genes (nonallelic interaction) undergo a lytic process leading to cell death. The accumulation of dying cells in the contact zone of two incompatible strains results in the formation of an abnormal contact named “barrage” (Rizet 1952).

Three nonallelic systems—het-c/het-e, het-c/het-d, and het-t/het-v—have been characterized in P. anserina (Bernet 1967, 1992). Genetic crosses between strains bearing incompatible genes at these loci lead to offspring containing incompatible combinations of the het genes in the same nucleus. Uninucleated spores of this geno-type can germinate and produce a homokaryotic mycelium, which, as the first consequence of incompatibility, stops growing a few hours after germination. The second consequence is a generalized lytic reaction of all the cells in the thallus, leading to the death of such self-incompatible (SI) strains (Bernet et al. 1973). However, these strains can “escape” from self-incompatibility as seen by the emergence of growing sectors. The mutations most often occur in the het genes, leading to a neutral interaction. However, strains that display a new phenotype, called MSI (modified self-incompatible), have been identified (Belcour and Bernet 1969; Bernet et al. 1973). Although the mycelium has few aerial filaments, the apical growth of these strains is restored. However, the lytic defect is only partly suppressed. Most of the MSI strains result from modifier (mod) mutations occurring in the unlinked mod-A locus (Belcour and Bernet 1969). All mutations identified at this locus are recessive. The mod-A1 mutation has no effect on allelic interactions but suppresses self-incompatibility resulting from coexpression of the het genes from the three nonal-
ileic het-c/ het-e, het-c/ het-d, and het-r/ het-v systems (Bel-cour and Bernet 1969; Bernet et al. 1973). No obvious phenotypical trait has been observed for mod-A1 mutants in a compatible background.

The MSI strains exhibit cold sensitivity (Bernet et al. 1973), which can be suppressed by mutations in the independent mod-B locus, where the mod-B1 mutation was previously identified (Bernet 1971). In MSI mod-A1 mod-B1 double mutant strains, lysis is suppressed (Bernet 1971; Labarere 1973). Although no mutant phenotype could be observed for the mod-B1 mutations in a compatible background, the mod-A1 mod-B1 double mutant exhibited a female sterility phenotype (Bernet 1971; Boucherie and Bernet 1974). This phenotype was the first evidence for a link between genes involved in incompatibility and development. However, the level of sterility is heavily dependent on growth conditions. This suggests that mod-A and mod-B are not strictly required for the differentiation processes but that they become essential for the successful development of the female organs in unfavorable conditions (Boucherie and Bernet 1980; Bernet 1992).

Incompatibility genes have been studied at the molecular level in N. crassa (Glass et al. 1990; Philley and Staben 1994; Sauge et al. 1996a; Sauge et al. 1996b) and P. anserina (Beuguer et al. 1994; Sauge et al. 1995; Espagne et al. 1997). Suppressors of heterokaryon incompatibility have been reported for N. crassa (Newmeyer 1970; Jacobson 1992; Vallani et al. 1994; Arangoza et al. 1994), but they have not yet been characterized at the molecular level. The mechanism of the lethality resulting from the interaction between het genes is still unknown. In addition, its relation to developmental processes remains unclear. Molecular characterization of the mod-A1 and mod-B1 modifier genes that are able to suppress incompatible interactions resulting from the three nonallelic systems in P. anserina and are involved in growth control and in female organ differentiation may help explain the link between incompatibility and development. In this study, we report on the isolation of the mod-A1 gene. No similarity with known proteins was observed for the mod-A1-encoded polypeptide. However, a proline-rich region with a potential SH3-binding domain was observed, which may suggest an involvement in protein-protein interactions.

MATERIALS AND METHODS

Fungal strains: P. anserina is a pseudohomothallic ascomycete. Its life cycle and the general methods used for genetic analysis have been described elsewhere (Rizet and Engelman 1949; Esser 1974). The specific heterokaryon-compatibility pattern of a strain can be determined by confrontation on cornmeal agar medium with appropriate tester strains of known genotype. When two strains are incompatible, a dense and unpigmented mycelial pad called a barrage is formed in the region of contact between thalli, where the mycelia form heterokaryons (Rizet 1952). Unless specified, the strains with the mod-A and mod-B mutations used in this study are all derived from the "F" geographical race (Bernet 1967) bearing the het-3 allele at the het-c locus. This gene is incompatible with the het-2 allele from the antagonistic het-e locus (Sauge et al. 1994). In the crosses involving the mod-A1 mod-B1 double mutant, the strains were used as male. Fertility tests were performed with strains grown in the dark for 5 days at 26°C on cornmeal agar medium or on synthetic medium (Loubradou et al. 1996). The plates were then transferred to the light; 3 days later, they were overlaid with a conidial suspension collected on a strain of the opposite mating type grown in the same conditions. The liquid was discarded, and the plates were checked after a few days for the development of perithecia.

Bacterial strain: Escherichia coli DH5α [F- endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 (lacZΔM15); Bethesda Research Laboratories, Gaithersburg, MD] was used for bacterial transformation and plasmid propagation. Bacterial plasmids used in this study were constructed by standard recombinant DNA techniques.

Transformation of protoplasts: Protoplasts were prepared and transformed as described previously (Bergerè and Barreu 1989). For transformation, the pMOcosX (Orbach 1994) bearing the hygromycin resistance gene (hph) as a selectable marker was used as a vector. Transformants were selected on synthetic medium supplemented with hygromycin B at 10 μg/ml. When cotransformation experiments were performed, 5 μg of the transforming plasmid was mixed with 2 μg of pMOcosX. To screen for the deletion of the mod-A gene, a mod-B1 strain bearing a leu1 mutation was constructed to serve as a recipient strain. The cloned leu1 gene (B. Turcq, unpublished results) was used as a selectable marker for transformation.

Nucleic acid isolation: General methods for nucleic acid analysis and plasmid construction were standard procedures (Sambrook et al. 1989). P. anserina DNA was prepared as described in Javerzat et al. (1993) or using the miniprep procedure described by Lecellier and Silar (1994). The library construction has been described elsewhere (Loubradou et al. 1996).

DNA manipulations and reverse transcription-PCR: Exonuclease III sequential deletions were performed using an Exolll/Mung bean nuclease deletion kit (Stratagene, La Jolla, CA) as recommended by the supplier. Sequencing was performed using the dideoxynucleotide chain termination method (Sanger et al. 1977) with the Sequenase reagent kit (version 2.0; United States Biochemical Corp., Cleveland) and [α-32P]dATP. The DNA sequence was established on both strands using clones sequentially deleted from both ends of the fragment and completed using specific synthetic primers derived from the available sequence.

Total RNAs and polyA+ RNAs were prepared as described (Turcq and Bégueret 1987). Northern blots were probed with the 7.4-kbp CiaI fragment from pCC3 (see Figure 1). For slot-blot analysis, different strains were grown onto cornmeal solid medium overlaid with cellophane disks, and RNAs were extracted as described earlier, from the mycelium scraped from the cellophane disks. Decreasing amounts of RNA (5, 1, 0.2, and 0.04 μg, respectively) were blotted as described (Sambrook et al. 1989) using a Hybri-slot manifold (BRL Life Technologies). For reverse transcription-PCR (RT-PCR), the 3′RACE kit for rapid amplification of cDNA ends (BRL Life Technologies, Gaithersburg, MD), based on the system described by Frohman et al. (1988) was used. The 3′-nucleotide AP primer containing a (dT)12 tail at the 3′ end was used to prime the cDNA synthesis with reverse transcriptase. For the first PCR, 1/20 of the CDNA reaction product was amplified using the AP primer and one of the sequence-specific
The second PCR was performed using 0.5 μL of the first PCR product as the source of DNA and two internal, sequence-specific primers. Primers used were as follows: OLI1 = 5’CGGCCACGAGACCGCAAC3’; OLI3 = 5’GAA CGAGATTGCAGACCGCC3’; OLIS = 5’CCGGCTGTCACG TG3’; OLIS = 5’GCCAGTAGGCTGAGGTC3’; OLIS = 5’ GTCCATGACGGCGAGATCGA3’; OLIS = 5’GAAGCA CGAGGTACACCA3’; OLIS = 5’GCATGCGGTAGCG3’ GTTGGCG3’; OLIS = 5’GGCCCCGCGCGTTGTC3’.

The PCR products were made blunt-end by treatment with Klenow polymerase cloned in the Eco RV site of the pBlue-script SK+ vector and sequenced as above.

RESULTS

Cloning of the mod-A gene: A library of 3000 clones (Loubradou et al. 1996) constructed in the pMOcosX cosmid (Orbach 1994) from DNA containing the wild-type mod-A gene was used to transform protoplasts of the het-c3 het-e3 mod-A1 mod-B1 strain. Hygromycin-resistant transformants were tested for their capacity to develop female organs. Three transformants that displayed perithecia after fertilization and were indistinguishable from the wild-type control were obtained. DNA was prepared from the fertile transformants and used either for in vitro packaging or for transformation of competent DH5α E. coli cells. The cosmid obtained from transfection following packaging were all identical in restriction pattern, but none of them were able to complement the defect in fertility of the het-c3 het-e3 mod-A1 mod-B1 strain. One cosmid, named pR4-5, obtained after direct transformation of E. coli, produced fertile transformants upon transformation of the recipient strain. Clai restriction of pR4-5 DNA resulted in only four insert fragments, which were cloned and assayed for ability to confer the fertile phenotype. The pCC3 plasmid containing a 7.5-kbp genomic Clai fragment retained this ability. The map of this insert is shown in Figure 1A.

To demonstrate that the cloned fragment was able to complement the mod-A1 phenotype in incompatibility, transformants obtained with pR4-5 cosmid were subjected to genetic and molecular analyses. Four transformants were purified by crossing with a het-c3 het-e3 mod-A1 mod-B1 strain isogenic to the recipient strain except for the mating type. For each strain, one homocaryotic, hygromycin-resistant strain was selected among the progeny (purified pR4-5 transformant) and then crossed with a het-c2 het-e1 mod-A1 MSI strain (het-c2 and het-e1 are incompatible genes); offspring were then analyzed (Table 1). SI and MSI strains were observed in the expected proportions, demonstrating that the cloned fragment was not only able to complement the sterile phenotype but also restored a wild-type behavior (i.e., self-incompatibility) identical to the wild-type mod-A allele.

Then, it was shown that the cloned gene was mod-A and not an extragenic suppressor of mod-A1. It has been previously observed that plasmids containing large inserts such as cosmids integrate with a high frequency at the resident locus (Picard et al. 1987). To locate the integration site of the cosmid, four independent pR4-5 transformants were crossed with a het-c2 het-e2 strain containing the wild-type mod-A gene. For one transformant, MSI strains were observed among the progeny, showing that integration occurred outside the mod-A locus; but for the three other transformants, no MSI strains were obtained, and SI strains were observed in 1/4 of the progeny—as expected for integration of the complementing gene at the mod-A locus. For at least two of these transformants, it was confirmed by Southern blot analysis that transformation occurred by integration in the genomic counterpart of the cloned fragment (result not shown), thus demonstrating that this fragment contained the mod-A gene.

Eight fertile strains issued from transformation with pCC3 were also crossed with a het-c2 het-e2 strain. As demonstrated by the presence of MSI strains in the progeny, six of the eight strains resulted from integration of pCC3 at an ectopic locus. DNA blot analysis confirmed this result. This excluded the possibility that fertile transformants might arise by reconstitution of a functional gene as the result of integration of the plasmid containing an incomplete wild-type mod-A gene in the mod-A1 mutant copy. This result therefore demonstrates that the mod-A gene contained in the cloned CC3 fragment is functional.

Analysis of the mod-A gene: Exonuclease III deletions were performed starting from the two ends of the insert of pCC3. Clones corresponding to progressive deletions of about 1000 bp were used for cotransformation experiments with pMOcosX, and the proportion of fertile transformants was determined. This allowed us to locate the complementing gene in a region corresponding to about three kbp. The pNRU plasmid, a subclone containing a 2828-bp NruI fragment (boxed in grey in Figure 1A), was able to complement the mod-A1 defect.

The insert of pNRU was sequenced on both strands, and an open reading frame (ORF) 687 amino acids long (nucleotides 438–2557) interrupted by a potential 55-bp intron (nucleotides 1510–1565) was identified (Figures 1 and 2). Complementation of the mod-A1 defect is lost when pNRU is restricted with BamHI or with EcoRV—which cut, respectively, at the beginning and at the end of the ORF (Figure 1). These data suggest that this ORF encodes the putative MOD-A protein.

Probably because the amount of the mRNA is insufficient, no transcripts corresponding to this ORF could be detected by Northern blotting. The mRNA transcribed from the mod-A gene was identified using an approach based on the 3’ RACE procedure (Frohman et al. 1988). The polyA+ mRNAs from the wild-type strain were used to synthesize cDNAs using the adapter primer (AP) designed to hybridize with polyA tails. After a first PCR with either the AP-OLI3 oligonucleotide pair or the AP-OLI5 pair (Figure 1), no specific band could be identified by ethidium bromide staining. When a Southern
Figure 1.—Map of the cloned DNA fragment containing the mod-A gene. (A) Restriction map of the Clai insert of pCC3. Restriction sites: BamHI (B), Clai (C), EcoRV (EV), MluI (Ml), Nrul (Nr), and Smal (Sm). Each of the horizontal lines above the map represents a subclone issued from an Exonuclease III deletion experiment. The right margin shows the percentage of fertile transformants obtained with pCC3 and with each of these subclones. The grey box delimits the Nrul fragment containing the mod-A gene. The mod-A ORF is schematized by the thick line bordered by the ATG and the polyadenylation site. The 55-bp intron is represented by the small box. The arrows represent the synthetic oligonucleotides used in the mRNA characterization (1 = OLI1; 3 = OLI3; 5 = OLI5; 6 = OLI6; 7 = OLI7; 8 = OLI8; 10 = OLI10; and AP = adapter primer). (B) Restriction map of the mod-A(leu1) construct used to generate the strain deleted for the mod-A gene. The left part of the pCC3 insert, delimited by the Clai and MluI restriction sites, was first deleted to generate a subclone in which mod-A was contained in a XbaI-ClaI fragment. The Smal fragment containing the ATG and 3/4 of the MOD-A NH₂-terminus was replaced by a 2.2-kbp blunt-ended HindIII-PstI fragment containing the P. anserina leu1 gene. Xb, XbaI. Arrows indicate the orientation of the genes.

 blot of the PCR products was probed with the Nrul fragment, one 1200-bp band (PCR with AP-OLI3 pair) and one 700-bp band (PCR with AP-OLI5 pair) were identified. No PCR product was detected in a control experiment where the reverse transcriptase had been omitted in the reverse transcription step. In contrast, an abundant specific PCR product was detected by ethidium bromide staining when using the same cDNA preparation as above and a pair of primers made up of AP and ROX, a primer specific for the het-c gene (Saupe et al. 1994). From the size of the bands detected in the Southern blot, it could be deduced that the 3'-OH end of this potential mRNA lies about 100-150 bases downstream from the TAG stop codon located at position 2555 (Figure 2). A second round of PCR was performed using as substrate an aliquot of the first PCR product issued from the AP-OLI3 pair and the OLI3-OLI6 or OLI5-OLI8 pairs as primers. For the OLI5-OLI8 pair, a fragment of the expected size (328 bp) was obtained. For the OLI3-OLI6 pair, which amplifies a region spanning a potential intron, the size of the fragment obtained was consistent with the size expected for a spliced mRNA (357 bp). Sequencing of this fragment showed that it corresponded to the sequence of the ORF in which the potential ORF, an RT reaction with polyA+ mRNAs as substrate was performed using the mRNA-specific oligonucleotide OLI6 as primer. A first PCR was then performed with the pair OLI1-OLI6. Again, no specific
band could be detected, but when the product of the first PCR was used as substrate for a second PCR with the OLI7-OLI10 pair as primer, it was possible to amplify a fragment of the expected size in which the intron has been removed.

The detection by RT-PCR of a cDNA corresponding to the whole potential mod-A ORF allowed us to demonstrate that this ORF is expressed. The 2828-bp NruI fragment (boxed in grey in Figure 1) containing this ORF will henceforth be referred to as the mod-A gene, and the polypeptide deduced from this ORF as the MOD-A putative protein.

MOD-A begins at position 438 (Figure 2), and the sequence around the initiation codon (CAACATGGA) follows the preference observed in fungi (i.e., an A or a purine at position –3) and has eight of the nine conserved nucleotides when compared with the fungal consensus sequence (Ballance 1986). Typical transcription signals can be detected in the 5′ upstream region. A potential TATA box is found at position 257 in a pyrimidine-rich region, 180 bp upstream from the ATG, and a CAAT box lies 20 bp upstream of this TATA box. In addition to these conventional promoter elements, three blocks containing 10–12 contiguous G or C are found 32, 100, and 256 bp upstream from the ATG.

The ORF is interrupted by a small intron. The splice junctions, or sites for lariat formation, are in good agreement with the consensus splicing sites 5′-G(A/T)GTA(G)A(C)GT(C/A)T(C) and A(G)C(T)AGC(G) 3′, and with the lariat sequence TGCTAAC/T C found for P. anserina introns (our unpublished results) or more generally for filamentous fungi (Ballance 1986). At the end of the ORF, the ATAAA sequence is present 130 bases downstream from the stop codon. This sequence may—according to the results of the RT-PCR, and in agreement with the situation generally found in eukaryotes—correspond to the polyadenylation signal.

The 687-amino-acid MOD-A predicted polypeptide is rich in leucine (9.3%) and proline (7.4%). Analysis of the polypeptide sequence did not give any information concerning the potential function of this protein, except that most proline residues are clustered in a short, proline-rich (30% proline) region (boxed in Figure 2). MOD-A putative protein.

TABLE 1

<table>
<thead>
<tr>
<th>Parental strains</th>
<th>Progeny expected if the cloned gene is mod-A + and integrates at the mod-A locus</th>
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<tbody>
<tr>
<td>pR4-5 Transformant MSI strain</td>
<td>3/4 Compatible (44)</td>
</tr>
<tr>
<td>(het-c3 het-e2 mod-A1 mod-B1 or mod-B +)</td>
<td></td>
</tr>
<tr>
<td>pR4-5 Transformant Wild-type strain</td>
<td>3/4 Compatible (66)</td>
</tr>
<tr>
<td>(het-c3 het-e3 mod-A1) (het-c2 het-e2)</td>
<td>1/4 SI (29)</td>
</tr>
<tr>
<td>(het-c3 het-e2 mod-A + mod-B1 or mod-B +) and</td>
<td></td>
</tr>
<tr>
<td>(mod-A + hygR)</td>
<td>mod-A1 mod-B1 or mod-B +)</td>
</tr>
</tbody>
</table>

For the parental strains, the genotype is italicized. The mod-A + hygR genes resulting from integration of the pR4-5 cosmid are enclosed in square brackets. mod-A + and mod-B + represent wild-type alleles. Incompatible combinations of het genes are underlined. The results of the crosses are indicated as the fraction of the progeny expected for each compatibility class if integration were to occur at the resident locus in the transformant: compatible, modified self-incompatible (MSI), and self-incompatible (SI). Experimental values for progeny corresponding to a cross with one transformed strain (T12-pR4-5) are indicated in parentheses. SI or MSI progeny containing either the mod-B + or the mod-B1 allele have the same aspect and have been pooled together. For incompatible progeny, the genotype is indicated below the expected results of the crosses.
Figure 2.—Nucleotide sequence and ORF of the mod-A gene. The numbers on the left refer to the nucleotide sequence, and those on the right refer to the amino acid sequence. The asterisk represents the stop codon. The elements of the promoter are lightly underscored. The intron sequence is heavily underscored, with italicized nucleotides corresponding to the conserved splice junctions and lariat sequences. The proline-rich regions are boxed, and the region showing alignment with the yeast verprolin repeated motif resembling SH3-binding domains is boxed in grey. The accession number is AF025289.

**P P X P**, with Ø representing hydrophobic residues and the two prolines in bold representing conserved residues that have been shown to play a critical role for binding to SH3 domains (Ren et al. 1993; Yu et al. 1994; Cohen et al. 1995).

The mod-A gene product is involved in differentiation of protoperithecia: The mod-A1 mutation does not confer a mutant phenotype in a compatible background, but when it is associated with mod-B1, female sterility is observed due to a defect in the formation of protoperithecia (Boucherie and Bernet 1974). This sterility is conditional and also greatly depends on the alleles present at the het-c and het-e loci (Boucherie and Bernet 1980). The phenotype of the het-c2 het-e2 strain used in this study was analyzed in different genetic combinations for mod-A and mod-B genes. For the mod-A1 strain compared with wild type, no difference is observed after exposure to the light, which induces the formation of protoperithecia (Figure 3A). As for mod-A1, the mod-B1 mutation alone does not alter fertility. The density offer a mutant phenotype in a compatible background, but when it is associated with mod-B1, female sterility is observed due to a defect in the formation of protoperithecia (Boucherie and Bernet 1974). This sterility is conditional and also greatly depends on the alleles present at the het-c and het-e loci (Boucherie and Bernet 1980). The phenotype of the het-c2 het-e2 strain used in this study was analyzed in different genetic combinations for mod-A and mod-B genes. For the mod-A1 strain compared with wild type, no difference is observed after exposure to the light, which induces the formation of protoperithecia (Figure 3A). As for mod-A1, the mod-B1 mutation alone does not alter fertility. The density offer a mutant phenotype in a compatible background, but when it is associated with mod-B1, female sterility is observed due to a defect in the formation of protoperithecia (Boucherie and Bernet 1974). This sterility is conditional and also greatly depends on the alleles present at the het-c and het-e loci (Boucherie and Bernet 1980). The phenotype of the het-c2 het-e2 strain used in this study was analyzed in different genetic combinations for mod-A and mod-B genes. For the mod-A1 strain compared with wild type, no difference is observed after exposure to the light, which induces the formation of protoperithecia (Figure 3A). As for mod-A1, the mod-B1 mutation alone does not alter fertility. The density offer a mutant phenotype in a compatible background, but when it is associated with mod-B1, female sterility is observed due to a defect in the formation of protoperithecia (Boucherie and Bernet 1974).
Suppressor of Incompatibility

mycelium is less dense, with very few aerial filaments, and the density of protoperithecia decreases (57 per cm²). In addition, these female organs are defective. Except for the trichogyne, which looks normal, they lack pili, which correspond to the nearby filaments that later form the envelope of the perithecium (Figure 3B). Such protoperithecia cannot form perithecia when fertilized with microconidia (Figure 4A). This defect is restored by transformation with the mod-A gene. The consequence of the defect on the development of female organs is also illustrated in Figure 4B. This figure shows the formation of perithecia resulting from cross-fertilization in the contact zone between het-c2 het-e2 strains of opposite mating types bearing different combinations at mod-A and mod-B loci and grown on minimal medium. Abundant perithecia are formed between the two wild-type strains. The crosses between mod-A and mod-B or between mod-A1 and wild-type strains are fertile. However, on this medium, it can be observed that the density of perithecia is clearly lower on the mycelium of the mod-A1 strain and that the perithecia are smaller. The density of perithecia in the cross between the mod-B1 and wild-type strain is comparable to that observed between two wild-type strains. In contrast, between the two mod-A1 mod-B1 strains of opposite mating type, no perithecia are formed, illustrating the sterile phenotype. It can also be observed that in the crosses between mod-A1 mod-B1 strains and any other strain, the perithecia never develop on the thallus of the mod-A1 mod-B1 strain.

The mod-A gene product is not essential, and its deletion confers the suppressor phenotype: It has been previously shown that the mod-A1 mutation can be suppressed by a nonsense suppressor (Béguer et al. 1973). This suggests that it is the loss of MOD-A function, rather than its alteration, that restores the growth of self-incompatible strains. To confirm this hypothesis, we constructed a strain deleted for the mod-A gene. The rationale was that the mod-A1 gene is not essential for growth and that the deletion of this gene in a strain containing the mod-B1 mutation will lead to female sterility. The Δmod-A(leu1) construct shown in Figure 1B was used for direct gene replacement. The major part of the mod-A ORF, including the ATG, was replaced by the P. anserina leu1 gene. The XbaI-ClaI DNA fragment containing the deleted mod-A gene was used to transform a leu1-1 het-c3 het-e3 mod-B1 recipient strain. Prototrophic transformants were tested for the development of female organs. Among 1200 transformants screened, 50 showed very low fertility or total sterility. Twenty-five of these transformants were used as males in a cross with the leu1-1 het-c3 het-e3 mod-B1 recipient strain. Five strains in which female sterility segregated with the leu1 marker, as expected for a gene replacement at the mod-A locus (Table 2), were selected for further analysis.

Southern analysis (results not shown) confirmed a correct gene replacement by the deleted construct in two of these strains, T19Δmod-A and T448Δmod-A. The prop-

Figure 3.—Defect in protoperithecia formation resulting from the mod-A1 mutation associated with the mod-B1 mutation. Mycelia were grown on solid cornmeal medium for 5 days and exposed to the light to induce the development of protoperithecia. (A) View of the mycelia at ×50 magnification. (B) Microscopic observation of defective protoperithecia of mod-A1 mod-B1 strain compared with those of the wild-type strain. Magnification is ×200.
A

wild-type  mod-A1  mod-B1

mod-A1  mod-B1  Tmod-A mod-B1  Amod-A  mod-B1

B

wild-type  mod-B1  mod-A1  mod-B1  wild-type

mat-  mat+  mat-  mat+  wild-type

Figure 4.—Fertility of different strains with various combinations of the mod-A gene. (A) View of the mycelia and perithecia (magnification is ×4) of strains grown on cornmeal medium and fertilized with microconidia as described in materials and methods. Tmod-A mod-B1 is a fertile strain issued from transformation of the mod-A1 mod-B1 strain with the pNRU plasmid containing the wild-type mod-A gene. Tmod-A mod-B1 is a strain deleted for the mod-A gene. (B) Cross-fertilizations. The mycelia were grown on synthetic solid medium until confrontation and exposed to the light until full development of perithecia resulting from fertilization in the contact zone between two thalli. Magnification is ×2.

tion. The phenotype of these strains was indiscernible from the mod-A1 mod-B1 mutant phenotype (Figure 4A). To analyze the effect of the deletion in an incompatible interaction, a cross with a Het-c2 Het-e2 wild-type strain was performed. In the progeny, SI and MSI strains were obtained in the expected proportions (Table 2). The MSI strains displayed the typical mod-A1 MSI phenotype, confirming definitely that the cloned gene is mod-A and that it is the absence of this gene product that restores the capacity of SI strains to grow. The strains deleted for the mod-A gene did not display an obvious phenotype in a compatible wild-type background.

**Constitutive overexpression of mod-A has no detectable effect:** We investigated whether the constitutive overexpression of the mod-A gene in a compatible background would affect protoperithecia growth and/or formation. The ORF between BamHI and NruI restriction sites (see Figure 1) was cloned downstream from the Aspergillus nidulans GPD constitutive promoter (Punt et al. 1987) to give the pGPD-MODA plasmid. This plasmid was used with the pMOcosX vector to cotransform the het-c3 het-e3 mod-B1 strain. Transformation with pGPD-MODA produces the same proportion of fertile transformants as pNRU, the plasmid containing the mod-A gene with its own promoter. This result demonstrates that the construct is functional in complementing the deficiency of the mod-A1 mutation. Using slot-blot analysis of total RNAs from the wild type, it was possible to detect a faint signal with the mod-A gene as probe. As a control, no signal was observed with RNA preparations of the mod-A deleted strains. A significantly stronger signal was observed in RNAs prepared from two pGPD-MODA transformed strains (Figure 5). Compared with the control het-c3 het-e3 mod-B1 strain or to the wild type, no differences in the growth rate and development of perithecia of these strains could be observed.

**DISCUSSION**

In P. anserina, mutations occurring in the mod-A locus are able to restore the growth of SI strains resulting from the combination of antagonistic incompatible genes from the three nonallelic Het-c Het-e, Het-c Het-d, and Het-r Het-v systems but do not suppress allelic interactions (Boucherie and Bernet 1969; Bernet et al. 1973). In the MSI strains, cell death is delayed but not suppressed, and lytic activities associated with cell death are not affected by mod-A mutations. These observations suggest that mod-A does not interfere with the cell death process, although this hypothesis cannot be fully excluded. The mod-A1 mutation was reported to interfere with the development of protoperithecia when associated with mutations in the mod-B locus (Boucherie and Bernet 1974). In an attempt to understand how the product of nonallelic incompatibility genes may inter-
### TABLE 2

Crosses performed to characterize the mod-A deleted strain

<table>
<thead>
<tr>
<th>Parental strains</th>
<th>Progeny expected if ([\Delta mod-A(\text{leu1})]) has replaced the resident locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta mod-A) deleted strain</td>
<td>Recipient leu(^-) strain</td>
</tr>
<tr>
<td>((\text{leu1-1 het-c3 het-e3 [(\Delta mod-A(\text{leu1})) mod-B1]} )</td>
<td>(leu1-1 het-c3 het-e3 [(\Delta mod-A(\text{leu1})) mod-B1])</td>
</tr>
<tr>
<td>(\Delta mod-A) deleted strain</td>
<td>Wild-type strain</td>
</tr>
<tr>
<td>((\text{leu1-1 het-c3 het-e3 [(\Delta mod-A(\text{leu1})) mod-B1]} )</td>
<td>(het-c2 het-e2 mod-A(^-) mod-B(^+))</td>
</tr>
<tr>
<td>((\text{leu1-1 het-c3 het-e3 [(\Delta mod-A(\text{leu1})) mod-B1]} )</td>
<td>(het-c3 het-e2 [(\Delta mod-A(\text{leu1})) mod-B1 or mod-B(^+)])</td>
</tr>
<tr>
<td>((\text{leu1-1 het-c3 het-e3 [(\Delta mod-A(\text{leu1})) mod-B1]} )</td>
<td>(het-c3 het-e2 mod-A(^-) mod-B1 or mod-B(^+))</td>
</tr>
</tbody>
</table>

For the parental strains, the genotype is italicized. The block resulting from replacement of the mod-A gene by the \(\Delta mod-A(\text{leu1})\) construct is enclosed in square brackets. leu\(^-\) and leu\(^+\) represent auxotrophy and prototrophy for leucine, respectively. \(mod-A\(^-\)\) and \(mod-B\(^+\)\) are wild-type alleles. The results of the crosses are indicated as the fraction of the progeny expected for each fertility phenotype for each class: compatible, modified self-incompatible (MSI), and self-incompatible (SI). The genotype is indicated below the expected results of the crosses. Experimental values for the progeny corresponding to a cross with the T19\(\Delta mod-A\) deleted strain are indicated in parentheses. MSI and SI progeny with the \(mod-B\(^1\)\) or the \(mod-B1\) allele have the same aspect and have been pooled together. Incompatible combinations of het genes are underlined.

fere with a gene that is involved in differentiation, we isolated the mod-A gene.

A genomic fragment was cloned by complementation of the sterility defect of the mod-A1 mod-B1 strain. It was shown that the cloned gene also complemented the mod-A1 mutation in the incompatibility reaction. It was confirmed by gene replacement that the cloned gene was the mod-A gene. The mod-A deleted strains are identical to the wild-type strain in a compatible background but exhibit female sterility when the deletion is associated with the mod-B1 mutation. This result is not surprising, since some mod-A mutations previously characterized as nonsense mutations (BégueR et al. 1973) conferred the same phenotype. As expected, the deletion of the mod-A gene also confers the MSI phenotype in an incompatible interaction. These results demonstrate (1) that the two functions, growth arrest in incompatible interaction and protoperithecia development, are controlled by mod-a and (2) that both the suppressor effect and the defect in fertility result from the absence of the mod-A gene product.

The ORF corresponding to mod-A was identified. The mRNA could not be detected by Northern blot in poly(A\(^+\)) mRNA preparations. As mod-A is always active in self-incompatibility, it should be expressed constitutively, at least at a low level. However, mod-A is also expected to act during female organ development in the fungal cycle. Its expression might then be regulated developmentally. However, increased expression at the moment of female organ differentiation was not detected (result not shown), and overexpression using a constitutive promoter produces fertile strains with the same phenotype as transformants obtained with the wild-type mod-A gene, suggesting that growth and differentiation are not affected by variation in the level of expression of the mod-A gene.

The predicted MOD-A protein deduced from the ORF sequence has no significant similarity to any known protein in the databases. However, sequence alignments with the yeast verprolin could be found for a small region rich in proline. The alignments involved a region in MOD-A that aligns with the various proline repeats of
verprolin, reported to exhibit similarity to SH3-binding motifs (Donnelly et al. 1993). The MOD-A domain contains a potential motif with proline at positions matching the conserved prolines of the SH3 ligand consensus sequence X P Ø P X P X (Yu et al. 1994). Such short proline-rich motifs have been characterized by their ability to bind SH3 domains of molecules acting in signaling processes such as protein kinases, phosphatases, and Ras-controlling proteins and are more generally involved in protein-protein interactions (Koch et al. 1991; Cohen et al. 1995). Recently, different observations suggested a role for proteins containing SH3 domains and SH3 ligand motifs in fungal morphogenesis. In the yeast S. cerevisiae, the defect in verprolin leads to large cells with distorted morphology (Donnelly et al. 1993), and the BEM 1 gene containing SH3 domain motifs plays a role in the polarized growth during budding and mating (Chenevert et al. 1992). A putative SH3 ligand motif has also been observed in the predicted mypl gene product of the dimorphic fungus Ustilago maydis, which was shown to be required for full expression of the mycelial phenotype during the transition from budding cells to filamentous growth (Gissan and Krosstad 1995). A gene required for nuclear migration and hyphal morphogenesis in N. crassa has recently been reported to contain proline-rich regions with potential SH3-binding domains (Vierula et al. and Mais 1997). This gene seems to play an important role in early events involved in the conidiation process.

Several recent results in our laboratory suggest an interconnection between incompatibility and signal transduction in P. anserina. Nonallelic genes of the het-c/ het-e incompatibility system were cloned. The het-c gene encodes a small protein displaying similarity to a glycolipid transferase protein, and its deletion drastically reduces the ascospore production (Saupé et al. 1994). The het-e gene codes for a large polypeptide with two domains: a functional GTP-binding motif and a domain with WD40 repeats, which suggests that this polypeptide can be involved in protein-protein interactions (Saupé et al. 1995; Espagne et al. 1997). It was then shown that an additional copy of the adenylate cyclase gene relieves developmental defects resulting from mutations in a gene involved in vegetative incompatibility (Loubradou et al. 1996). More recently, the mod-E gene, in which mutations suppress some developmental defects, was shown to partly restore the growth of het-c/het-v SI strains. This mod-E gene (Loubradou et al. 1997) was characterized as a member of the HSP90 protein family, which is implicated in many cellular processes involving signal transduction (Lindquist and Craig 1988). The presence of potential SH3-binding motifs in the MOD-A protein may also suggest a potential role in signal transduction during female organ formation. This signal may transfer the information required to control differentiation of protoperithecia from quiescent mycelial cells, where fruiting bodies form. However, the effect of the absence of a signal can be observed only when mod-B1 mutation is present. This suggests a dual action of these two genes in the same pathway or involvement in parallel pathways. An inhibition of apical growth might be a prerequisite to successfully differentiate a female organ from quiescent cells. A tempting interpretation has been proposed by Benett (1992). As reported previously, the level of sterility of the mod-A mod-B1 strain is conditional, depending on the temperature, the medium, or the age of the mycelia (Boucherie and Benett 1980). This might suggest for MOD-A a role in particularly limiting conditions—such as total exhaustion of nutrients, for example. To mobilize all the residual growing potential for the formation of fruiting bodies, a growth arrest might be coupled with a partial lysis of hyphal cells in the region surrounding the protoperithecia. This lysis, which is thought to generate the nutrients necessary for the achievement of female organ development, would be controlled by the mod-B gene. Without these two control steps—which are presumably interconnected—the development aborts, leading to defective protoperithecia. Arguing in favor of this hypothesis are the facts that sterility of mod-A mod-B1 strains can be fully suppressed by addition of various amino acids to the medium (Boucherie and Benett 1980) and that sterility is never observed on complete medium.

The question remains how these control steps can be linked to three nonallelic het systems. First, these het genes are probably redundant (HET-E and HET-D polypeptides are very similar; unpublished result) and can be presumed to act in the same pathway. Second, the observation that the level of female sterility of the mod-A mod-B1 strains is strongly dependent on the combination of compatible het genes at het-c, het-e, het-d, het-r, and het-v loci (Boucherie and Benett 1980) led Benett (1992) to propose a potential role for the nonallelic het genes in the control of female organ development. It can be speculated that this control involves MOD-A. Then, in case of an incompatible interaction of nonallelic het gene products, an abnormal signal leads to a cellular catastrophe resulting from the deregulation of the two pathways controlling growth and cell lysis, respectively. Mutations in mod-A suppress the effect of this signal transmission, thus allowing the growth, and dominant mutations in mod-B relieve the downstream process of cell lysis.

The functionality of the potential SH3 ligand of the MOD-A polypeptide is now under investigation, and its potential interaction with het-e and/ or het-c gene products will be assessed. Determination of the exact function of HET-C and HET-E in the fungus and cloning of the mod-B gene will help to explain the mechanism connecting incompatibility and female organ differentiation.

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**LITERATURE CITED**


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