ami1, an Orthologue of the *Aspergillus nidulans apsA* Gene, Is Involved in Nuclear Migration Events Throughout the Life Cycle of *Podospora anserina*

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ABSTRACT

The *Podospora anserina ami1-1* mutant was identified as a male-sterile strain. Microconidia (which act as male gametes) form, but are anucleate. Paraphysae from the perithecium beaks are also anucleate when *ami1-1* is used as the female partner in a cross. Furthermore, in crosses heterozygous for *ami1-1*, some crozier cells are uninucleate rather than binucleate. In addition to these nuclear migration defects, which occur at the transition between syncytial and cellular states, *ami1-1* causes abnormal distribution of the nuclei in both mycelial filaments and asci. Finally, an *ami1-1* strain bearing information for both mating types is unable to self-fertilize. The *ami1* gene is an orthologue of the *Aspergillus nidulans apsA* gene, which controls nuclear positioning in filaments and during conidiogenesis (at the syncytial/cellular transition). The ApsA and AMI1 proteins display 42% identity and share structural features. The *apsA* gene complements some *ami1-1* defects: it increases the percentage of nucleate microconidia and restores self-fertility in an *ami1-1 mat+* (*mat−*) strain. The latter effect is puzzling, since in *apsA* null mutants sexual reproduction is quite normal. The functional differences between the two genes are discussed with respect to their possible history in these two fungi, which are very distant in terms of evolution.

NUCLEAR migration and positioning are fundamental and ubiquitous processes. Numerous observations have shown that nuclear movements are essential for growth and development in both lower and higher eukaryotes. For instance, the migration of the male pronucleus within the animal oocyte after fertilization, together with movements of the female pronucleus, are essential events for nuclear fusion. Similarly, nuclear movements are required in yeasts after mating to achieve caryogamy. Later in development, nuclear positioning is especially important for asymmetric cell division in plant and animal embryos. Similarly, in *Saccharomyces cerevisiae*, migration of the mother cell nucleus to the bud neck before mitosis ensures that the daughter cell will contain one of the two sister nuclei. An interesting scenario is observed in early development of insects, the paradigm of which is Drosophila: after several mitotic divisions of the zygotic nucleus (without cytokinesis), there is a massive migration of nuclei from the center of the embryo to a subcortical position at which cellularization occurs. This situation, which requires a transition between a syncytial and a clearly cellular state, is not specific to insect development: it is also encountered during vegetative and sexual development in some plants and in filamentous fungi.

In the last decade, many studies associating cytological, biochemical, and genetic approaches have been devoted to nuclear movements. In this research area (as in others), yeasts and filamentous fungi have played key roles as model systems. Several genes required for proper nuclear distribution during vegetative growth have been characterized in *Aspergillus nidulans* and *Neurospora crassa* (Morris *et al.* 1995; Fischer 1999 for reviews). With respect to their functions, these genes (which have been named *nud* in *A. nidulans* and *ro* in *N. crassa*) can be grouped into three classes. Some encode components of cytoskeletal motors: *nudA* (Xiang *et al.* 1994), *nudG* (Beckwith *et al.* 1998), *ro1* (Plamann *et al.* 1994), *ro3* (Tinsley *et al.* 1996), and *ro4* (Plamann *et al.* 1994; Robb *et al.* 1995). Others probably fulfill regulatory roles: for instance, *nudC* and *nudF* (Osmani *et al.* 1990; Xiang *et al.* 1995). Still others embody poorly understood functions: for instance *ro2* (Vierula and Mais 1997), *ro10*, and *ro11* (Minke *et al.* 1999). Some of these genes have orthologues in higher eukaryotes. The role that filamentous fungi can fulfill in the elucidation of complex processes in higher eukaryotes, and in a comprehensive view of the evolutionary history of eukaryotes, can be illustrated by *nudF*, orthologues of which have been found in Drosophila and mammals (Reiner *et al.* 1993; Xiang *et al.* 1995; Cunniff *et al.* 1997; Morris *et al.* 1997). One remarkable point is that *nudF*, essential for vegetative growth in *A. nidulans*, has been identified as a gene required in a mammalian developmental program. In fact, mutations in *LIS1* (the human orthologue of *nudF*) lead to diseases characterized by malformations in the cerebral cortex (Reiner *et al.* 1993). The conventional explanation of these defects

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assumes a problem in neuronal migration (Barth 1987). However, Morris *et al.* (1998) propose that *LIS1* might be involved (as is its *A. nidulans nudF* orthologue) in the control of nuclear movements that occur early in cerebral development and have been implicated in cell fate determination (Morris *et al.* 1998 and references therein).

Although the *nud* and *ro* mutations can impair asexual and sexual development, the primary role of the relevant genes is at the level of vegetative growth. Other genes are more specifically involved in nuclear migration events linked to development. In *A. nidulans*, Clutterbuck (1994) identified two genes, *apsA* and *apsB*, required for conidiation (asexual sporulation) at a very precise stage of conidiophore development, corresponding to the transition between the syncytial and cellular states. In the mutants, the structures that are normally uninucleate do not (with rare exceptions) contain a nucleus. The *apsA* and *apsB* genes were cloned and null mutants were constructed: they show the same phenotypic properties as do the original mutants, *i.e.*, clustering of nuclei in the filaments and a specific defect of nuclear migration during the conidiation process (Fischer and Timberlake 1995; Suelmann *et al.* 1998). The ApsA protein was localized at the cytoplasmic membrane and the ApsB protein was localized in the cytoplasm (Suelmann *et al.* 1997, 1998). ApsB shows sequence similarity to trichohyalin, which is thought to interact with intermediate filaments in mammals (Tarcsa *et al.* 1997 and references therein). *apsA* has a structural orthologue in *S. cerevisiae*, *NUM1*, whose mutations impair nuclear migration to the bud before (but not after) mitosis (Kormanec *et al.* 1991; Revardel and Aigle 1993; Farkasovsky and Küntzel 1995).

Although the data as a whole focus on a regulatory role of ApsA and ApsB in nuclear migration / positioning processes, their precise functions remain unclear. In this study, we identify and analyze *ami1* (anucleate microconidia), the orthologue of *apsA* in *Podospora anserina*, a filamentous ascomycete very distant from *A. nidulans* in terms of evolution. While *apsA* seems to be involved mainly in nuclear migration / positioning processes during conidiation, *ami1* exhibits broader functions, especially at all steps requiring nuclear movements during sexual reproduction.

MATERIALS AND METHODS

***P. anserina* strains and media:** *P. anserina* is a heterothallic filamentous ascomycete whose life cycle and general methods for genetic analysis have been described (Rizet and Engelmann 1949; Esser 1974). All strains are derived from the wild-type S strain (Rizet 1952). We also used another wild-type strain called s, which differs from the S strain in the allele present at the *s* locus (Bernet 1965) later named *het-s* (Deleu *et al.* 1993). The *193* mutant strain exhibits white mycelium, perithecia, and ascospores (Picard 1971). The *incA* mutation (first named *I* for *incoloris*) is highly pleiotropic (see results; Rizet and Engelmann 1949; Prillinger and Esser 1977; Bernet 1986, 1988). The *cro1-1* mutation leads, when homozygous, to giant plurinucleate croziers (Simonet and Zickler 1978; Berteaux-Lecellier *et al.* 1998). Mutants of the *FMR1* and *SMR2* (*mat*−) genes were described by Arnaise *et al.* (1997) and the Δ*mat* (*mat*−) strain was described by Coppin *et al.* (1993). The culture and spore germination media have been reviewed recently by Berteaux-Lecellier *et al.* (1995).

Counting of microconidia and perithecia: The relevant strains were grown on petri dishes containing minimal synthetic medium (M2) and incubated at 27° in the dark. The microconidia were recovered at different times (from 5 to 30 days) by washing the surface of the mycelia with 1.5 ml of sterile water. This permitted the recovery of 1 ml of microconidial suspension, which was counted by microscope with a hemacytometer. To test their fertilization ability, 1 ml of microconidial suspension (after dilutions when required) was spread on wild-type mycelia used as female partners, which were previously grown on M2 medium at 27° for 3 days in the light to allow formation of female organs. Perithecia were counted 5 days after fertilization.

Cosmids, plasmids, and bacterial strains: Cosmids N9 and N10 are taken from a *mat*− library (Picard *et al.* 1991). Cosmid U1 is from a *mat*+ library constructed by Turcq *et al.* (1990). The *ami1* gene was sequenced using either cosmids or subclones in pUC18, pBluescript SK(+), or KS(+) (Stratagene, La Jolla, CA). Cosmid and plasmid preparations were performed in *Escherichia coli* DH5α (Hanahan 1983) or CM5α (Camonis *et al.* 1990). The pMOcosX plasmid was used in cotransformation experiments: it carries the bacterial hygromycin-resistant gene under the control of the *cpc1* promoter of *N. crassa* (Orbach 1994). The plasmid carrying the *A. nidulans apsA* gene (a kind gift of Dr. R. Fischer) is pRF7, which has been described in Fischer and Timberlake (1995).

Transformation procedures: Transformation experiments were performed as previously described (Picard *et al.* 1991), except that the protoplasts were made with Glucanex (Novo Nordisk Ferment AG) rather than with Novozym. With cosmids N9 and N10, the recipient strain was *ami1-1 leu1-1*, since auxotrophy for leucine due to the *leu1-1* mutation is suppressed by the *su8-1* tRNA suppressor carried by these cosmids (Debuchy and Brygoo 1985). In all other cases (cotransformation experiments with pMOcosX), the recipient was the *ami1-1* strain and transformants were selected as hygromycin-resistant clones.

Physical localization of the *ami1* gene: The gene was first localized in the vicinity of the *mat* locus through complementation of the *ami1-1* mutant by cosmids encompassing this region. A precise localization of the gene on the N9 cosmid was then obtained according to the procedure developed by Turcq *et al.* (1990). Briefly, the cosmid was digested with one of several restriction enzymes. Each restriction mixture was used to transform the *ami1-1* strain using the pMOcosX reporter vector as a selective marker. Hygromycin-resistant transformants were tested for their ability to fertilize the wild-type strain used as the female partner. This method demonstrates which enzymes inactivate or fail to inactivate the gene.

Genetic and molecular attempts to obtain an *ami1* mutation in the *mat*− context: The *ami1* gene is tightly linked to the *mat* locus (∼10 kb), and recombination events between the *ami1-1* mutation and the *mat* locus were not obtained (among >100 asci analyzed). More importantly, cloning of the entire *ami1* gene on a DNA fragment whose size would have permitted deletion of the gene and then obtaining a null mutant through gene replacement could not be achieved. In fact, in all cases the expected fragments were rearranged. Lastly, since *ami1-1* is a suppressor of the *incA* mutant female sterility, UV mutageneses on an *incA mat*− strain were performed, without

success. This failure might be due to the fact that the *incA* mutation is dominant and that *incA* nuclei dominate any nuclei leading to an incA$^+$ phenotype.

Sequencing: The *ami1* gene was sequenced on both strands with an automatic sequencing machine (373A DNA sequencer, Applied Biosystems, Foster City, CA) by the method of DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Universal primer and synthetic oligonucleotides were used to start the reactions. The sequence was performed either on cosmids or subcloned fragments of the *ami1* gene. PCR amplification on genomic DNA from a mutant strain provided the templates for sequencing reactions. Total DNA was extracted by a miniprep method (Lecellier and Silar 1994). The amplified fragments were prepared for direct sequencing according to Rosenthal *et al.* (1993).

Northern (RNA) blot analysis: Total RNA was isolated from mycelia as described by Lockington *et al.* (1987), glyoxal treated, and separated on a 1% agarose gel (Sambrook *et al.* 1989). Northern blots were performed using standard techniques, with a probe of a 4.6-kb *Eco*RI fragment containing the central part of the gene. Poly(A) RNAs were obtained with a QIAGEN kit (oligotex mRNA minikit) and then treated with DNase and used as templates for reverse transcriptase (RT)-PCR experiments (TITAN one tube RT-PCR kit, Boehringer Mannheim, Mannheim, Germany). cDNA amplification was performed using synthetic oligonucleotides. The amplified fragments were prepared for direct sequencing as described above.

Heterologous hybridizations: Genomic DNA samples from *Ascobolus immersus*, *A. nidulans*, *S. cerevisae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, and *Homo sapiens* were kindly provided, respectively, by A. Grégoire, B. Felenbock, M. Bolotin-Fukuhara, M. Sipiczki, M. Kreis, and J. Levilliers; *Sordaria macrospora* is the St. Ismier FGSC 4818 strain. DNAs were digested and separated on a 0.7% agarose gel. The transfer was performed on a nylon membrane (Amersham Corp., Piscataway, NJ). After hybridization at low stringency (37°), using the procedure described by Church and Gilbert (1984), the membrane was washed at 45°, in 2× SSC. Two 2-kb PCR fragments corresponding to the 5′ and 3′ regions of the *ami1* gene were used as probes, except for *S. macrospora* DNA, which was hybridized only with the 3′ fragment. Control experiments were performed using the *P. anserina AS4* gene encoding the evolutionarily well-conserved eEF1A elongation factor (Silar and Picard 1994).

Light microscopy preparations: Nuclei were visualized through 4,6-diamidino-2-phenylindole (DAPI) staining (0.5 μg/ml). Mitochondria were stained with the mitochondrion-specific dye 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI; Sigma, St. Louis) using the procedure described by McConnell *et al.* (1990) and adapted for *P. anserina* by Jamet-Vierny *et al.* (1997). Microtubules were visualized, according to Thompson-Coffe and Zickler (1992), with a monoclonal anti-β-tubulin (Amersham, France) at a dilution of 1:1200. As secondary antibody we applied the FITC-labeled goat anti-mouse IgG antibody (Jackson Immunoresearch, West Grove, PA) at 1:100. Rosettes of asci were fixed in fresh Lu's (1967) fixative (butanol, propionic acid, and 10% aqueous chromic acid; 9:6:2 volume). After a 10-min hydrolysis in 1 n HCl at 70°, asci were stained in one drop of ferric acetate solution mixed on the slide with two drops of 2% hematoxylin.

RESULTS

The *ami1-1* mutant differentiates anucleate microconidia: The *ami1-1* mutant was serendipitously obtained during the systematic analysis of *mat*+ transgenic strains carrying a cosmid encompassing the *mat*− region (Picard *et al.* 1991). It was initially characterized as a male-sterile mutant, according to the following procedure: when two strains of opposite mating types are grown on the same petri dish, both produce male gametes (microconidia) and female organs (protoperithecia) but are self-sterile. To fertilize both strains with compatible microconidia, 1 ml of sterile water is poured on the mycelia and the dish is gently shaken to remove microconidia and distribute them over the surface of the cultures. As shown in Figure 1, this simple test defines the ability of a strain to be a male and/or a female partner. The *ami1-1* mutant clearly exhibits a female-fertile but male-sterile phenotype (compare Figure 1, A and B). Dozens of such crosses were performed: the number of perithecia observed on the female partner of *ami1-1* was

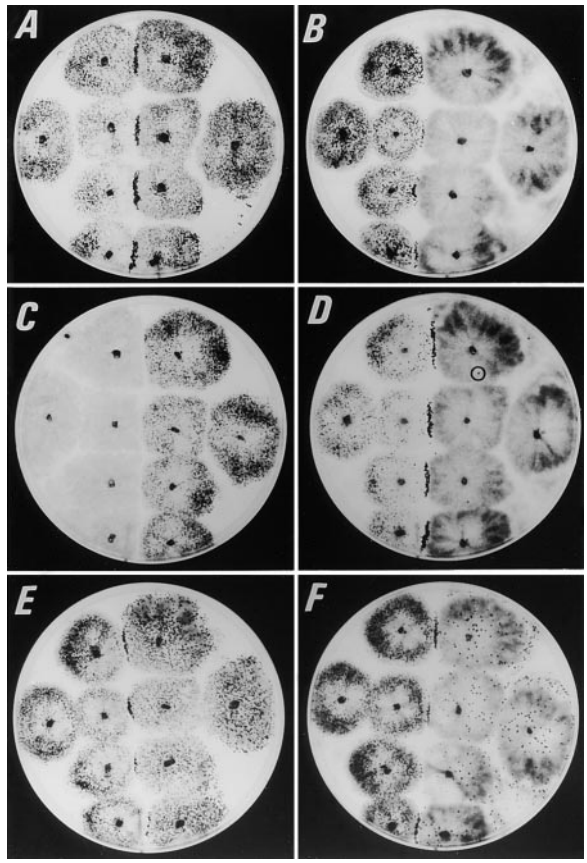

Figure 1.—The *ami1-1* mutant displays a male-sterile phenotype. Abilities of the relevant strains to act as male and female partners were tested according to the procedure described in the text. Crosses are as follows (with the first strain on the left of the petri dish): (A) wild type × wild type; (B) *ami1-1* × wild type; (C) *incA* × wild type; (D) *ami1-1 incA* × wild type; (E and F) two *ami1-1* transgenic strains carrying the *A. nidulans apsA* gene crossed to the wild type [(E) *ami1-1*(*apsA-1*); (F) *ami1-1*(*apsA-12*)]. The circle in D indicates one of the very few perithecia that can be observed when *ami1-1* is used as a male partner in a cross. Each strain is represented by five thalli (colonies). (*apsA-1*) and (*apsA-12*) are two integration events of the *A. nidulans apsA* gene.

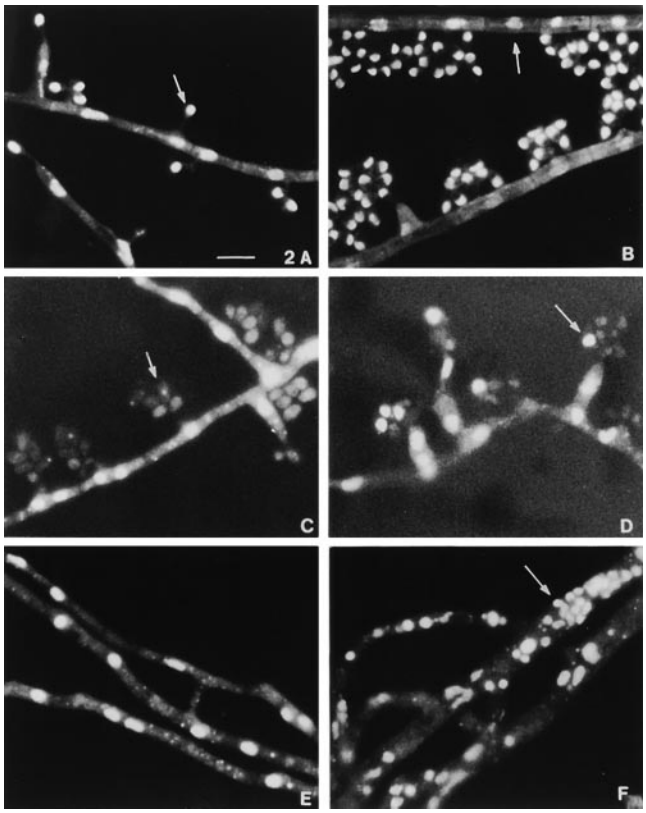

Figure 2.—The *ami1-1* mutant differentiates anucleate microconidia and exhibits abnormal distribution of nuclei in the mycelium (stained by DAPI). (A) Wild type. In Podospora, conidia are formed directly from the mycelium (arrow). (B) *incA* mycelia produce especially large numbers of conidia from each cell (arrow points to a nucleus). (C) Double mutant *incA ami1-1* mycelium with anucleate conidia (arrow, compare with B). (D) *ami1-1* transgenic strain carrying the *A. nidulans apsA* gene [*ami1-1* (*apsA-1*)], showing a mixture of nucleate (arrow) and anucleate conidia. (E) Wild-type mycelium with regularly spaced nuclei. (F) *ami1-1* mycelium with irregular distribution of the nuclei (arrow). Bar, 5 μm.

mainly 0 and never exceeded 3 or 4, while >1000 perithecia were formed in control crosses.

The *ami1-1* defect could have two possible explanations: either the mutant is aconidial, *i.e.*, unable to differentiate microconidia, or mutant microconidia are nonfunctional. Cytological analyses of *ami1-1* cultures showed that the mutant was able to form microconidia, but that almost all were anucleate (Figure 2): less than 10^{-3} contained a nucleus. Moreover, the distribution of nuclei throughout mycelial filaments was also abnormal: while uniformly distributed along the length of the filaments in wild-type mycelium, they were mainly clustered in the mutant, leaving large gaps in the mycelium (Figure 2). However, mitoses are normal in the mutant. Although the same clustering of nuclei was observed in the female organs, their development was not hindered. Mutant trychogynes do not differ from those of the wild type: in both cases, these specialized filaments contain two to three nuclei (data not shown). To determine if nuclear clustering corresponded to a possible general defect in organelle distribution in the mutant, we stained the mitochondria with DASPMI and followed their distribution in growing mycelium and microconidia. The mutant mitochondria do not significantly differ from the wild-type organelles with respect to size, number, and distribution. Furthermore, anucleate microconidia contain the normal average number of mitochondria, *i.e.*, from one to three (data not shown).

The rare perithecia observed when *ami1-1* is used as a male partner could be explained by its very weak leakiness (production of 10^{-3} nucleate microconidia). However, perithecia could also arise from mycelial fragments resulting from *ami1-1* cultures and acting as male gametes. To ascertain whether or not *ami1-1* filaments could act as male gametes, two tests were performed. First, we crossed *ami1-1* and the *193* mutant strain, which develops white perithecia, by confronting their mycelia on a petri dish. As shown in Figure 3, A and B, the presence of white perithecia all along the confrontation line (where the two mycelia meet) shows that *ami1-1* is able to act as a male partner. To ensure that this could not be explained by complementation of the mutant through heterocaryosis in the confrontation area, we performed a second set of crosses. When mycelial filaments of the wild-type s strain meet those of the S strain (from which *ami1-1* was issued), incompatibility results in the failure to form heterocaryotic filaments via hyphal fusions: fusion between incompatible hyphae leads to death of the heterocaryotic cells and causes the formation of a "barrage" or barrier between the two strains (Rizet 1952). As shown in Figure 3A, the confrontation of *ami1-1* S and *ami1*$^+$ s strains gives rise to a barrier, on both sides of which perithecia have developed. This confirms that *ami1-1* filaments can indeed act as male gametes in a cross. In fact, in this case, heterocaryosis is prevented and the *ami1-1* strain cannot be complemented for production of functional microconidia. If *ami1-1* filaments were unable to act as male gametes, perithecia would have been observed on the *ami1-1* strain but not on its *ami1*$^+$ female partner.

To better understand the *ami1-1* conidiation defect, we constructed double mutant strains with the *incA* mutation, known to increase the number of microconidia by a factor of 1000 as compared to young wild-type cultures (Table 1). A massive production of microconidia can be observed after a few days of culture of the *incA* mutant while, with wild-type strains, several weeks are required to produce similar amounts (Table 1). The *incA* mutant is also female sterile and displays a defect in mycelial pigmentation (Figure 1C). The phenotypic properties of the *ami1-1 incA* double mutant were examined using the tests previously performed for the two single mutants. As shown in Figure 1D and Table 1, the double mutant strain is male sterile: the number of perithecia observed for the female partner is similar to the number observed with the single mutant *ami1-1*.

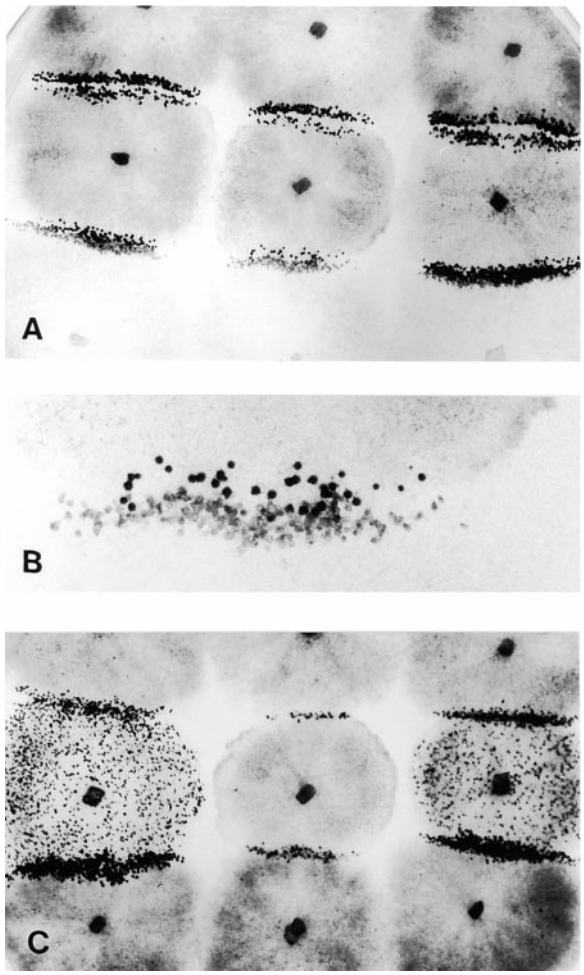

Figure 3.—Mycelial filaments of *ami1-1* can act as male gametes but *ami1-1 mat*+ (*mat*−) strains are self-sterile. (A) Confrontations of s (top testers) and *193* (bottom testers) with S (middle left), *ami1-1* (middle center), and *ami1-1* (*apsA-1*) (middle right). Note that in the three cases perithecia develop on both sides of the barrage and that white perithecia (issued from *193* female organs) are formed in the test with *193*. (B) Magnification of the confrontation between *ami1-1* and *193*, which demonstrates clearly the black (female *ami1-1*) and white (female *193*) perithecia. (C) Confrontations of S *mat*+ (top testers) and S *mat*− (bottom testers) with *ami1*$^{+}$ *mat*+ (*mat*−) (middle left), *ami1-1 mat*+ (*mat*−) (middle center), and *ami1-1 apsA-1 mat*+ (*mat*−) (middle right). Note that *ami1-1 mat*+ (*mat*−) is self-sterile although it gives rise to perithecia with the two (*mat*+ and *mat*−) testers and that *apsA-1* restores self-fertility.

Moreover, the double mutant microconidia remain anucleate (Figure 2). Thus, the *incA* mutation does not suppress the *ami1-1* defect. In contrast, the *ami1-1* mutation acts as a suppressor of the *incA* mutation with respect to female sterility (compare Figure 1, C and D) and massive production of microconidia. In fact, as seen in Table 1, the number of microconidia produced by the *ami1-1 incA* double mutant increases with the time of culture, but never reaches that observed in both *incA* and wild-type strains.

To complete this analysis, we attempted a search for suppressors of the male sterility phenotype of the *ami1-1* mutant. However, this was hampered by the spontaneous appearance of mutations leading to an *incoloris*-like phenotype able to partially suppress the mutant defect (S. Arnaise, personal communication).

The *ami1* gene is involved in sexual reproduction: Crosses between *ami1-1* and the wild type are fertile, although ascospores are expelled with a slight delay (∼1 day) compared to wild type × wild-type crosses (data not shown). However, 36–38% of the asci formed in those heterozygous crosses are abnormal: the nuclei formed after meiosis and postmeiotic mitosis are more or less randomly distributed in the asci, leading to abnormal ascospore delimitation and/or formation. Thus, *ami1-1* is semidominant. We examined the microtubule (MT) cytoskeleton organization during both ascus development and sporulation. The densities of the cortical array organized by the apical microtubule organizer center during the growth period of the ascus, as well as the nuclear-envelope-associated MTs, suggested to serve in positioning nuclei before and after division (Thompson-Coffe and Zickler 1992), are both normal when compared to wild-type crosses. Spindle pole bodies, spindle elongation, and astral MTs are also normal. During wild-type postmeiotic mitosis (PMM), the four spindles are in two widely separated pairs: thus, extensive nuclear movements occur before ascospores are formed. In ∼40% of the asci formed in the *ami1-1* × wild-type crosses, the four PMM spindles are either located in the middle of the ascus or randomly spaced, hindering the proper migration of the nuclear pairs before ascospore formation (data not shown). We also noticed a maternal effect of the mutation in all crosses involving *ami1-1*. The paraphysal hyphae, which are formed between the asci and at the base of the perithecial beak, show an abnormal nuclear distribution. This is especially striking in the beak paraphysae, which are normally formed by 5–10 uninucleate, short, and regularly sized cells emerging from basal plurinucleate cells (Figure 4A). In the mutant perithecia, these cells are irregular in size. Moreover, those cells located near the basal cell of the paraphysae maintain a plurinucleate state, while apical cells are anucleate (Figure 4B), thus suggesting a nuclear migration defect in this maternal tissue.

To further examine the possible role of the *ami1* gene in sexual reproduction *sensu stricto*, crosses in which the *ami1-1* mutation was homozygous were required. The original mutation was obtained in a *mat*+ context and genetic and molecular attempts to obtain a *mat*− strain bearing an *ami1* mutation have as yet been nonproductive (see materials and methods). We thus attempted to circumvent this problem in two ways. First, we examined the effect of the *ami1-1* mutation when heterozygous in crosses involving particular genetic backgrounds. Second, we constructed a strain allowing crosses in which all genes (including the *ami1-1* allele)

TABLE 1

Microconidia production by the *ami1-1* mutant and other relevant strains

Strains[a]	No. of microconidia[b]			No. of perithecia[b]	
	5 days[c]	20 days	30 days	4 days	15 days
Wild type	$\leq 10^4$	5×10^6	2×10^7	84 ± 5 (10^2)	28 ± 3 (10^4)
incA	6×10^7	8×10^7	8×10^7	104 ± 10 (10^5)	154 ± 11 (10^5)
ami1-1	$\leq 10^4$	4×10^5	5×10^6	1.5 ± 0.5[d]	11 ± 5[d]
ami1-1 incA	$\leq 10^4$	1.5×10^6	2×10^6	0[d]	50 ± 4[d]
ami1-1 (*apsA-1*)	$\leq 10^4$	3×10^5	2×10^6	52 ± 5 (10^1)	54 ± 9 (10^2)
ami1-1 (*apsA-12*)	$\leq 10^4$	ND	10^6	12 ± 0.5[d]	168 ± 10[d]

ND, not determined.

[a] (*apsA-1*) and (*apsA-12*) correspond to two integration events of the *A. nidulans apsA* gene.

[b] Microconidia were recovered at different times (from 5 to 30 days) by washing the surface ($\sim$60 cm^2) of one petri dish of each culture. They were counted using a hemacytometer chamber. The numbers correspond to 1 ml of suspension. Perithecia were counted after fertilization of the wild-type strain used as the female partner with 1 ml of microconidia suspension of the relevant strain. The numbers are the mean values of three to four petri dishes with, in parentheses, the dilution factor of the microconidia suspension (standard deviation).

[c] Values in the range of 10^4 (and less) cannot be reliable since they correspond to the lowest limit of the chamber. Otherwise, we did not have confidence in concentrated microconidia suspensions obtained by centrifugation because they gave variable results from one strain to the other: in particular, it was observed that in strains carrying the *ami1-1* mutation, the pellets contained highly enriched populations of nucleate microconidia.

[d] Undiluted.

were artificially homozygous, with the exception of the mating-type genes.

With respect to the initial purpose, we employed the *cro1-1* mutation and mutations in the *mat*− genes. An homozygous *cro1-1* mutation leads to a defect in dicaryotic cell formation preceding meiosis: rather than binucleate, the crozier cells are multinucleate, as though the *cro1-1* mutation hampers the transition between syncytial and cellular stages (Simonet and Zickler 1978; Berteaux-Lecellier *et al.* 1998). It is not clear whether this default is an initial failure in nuclear migration, or a defect in septum formation after cellularization. To determine if the *ami1-1* mutation (when heterozygous) could enhance or suppress *cro1-1* effects, a double mutant strain was constructed and the *cro1-1 ami1-1* × *cro1-1 ami1*$^+$ cross was compared to a *cro1-1* × *cro1-1* cross. The differences between the two crosses appeared to be very slight. However, one difference was noticeable: asci with four spores were more common in the *ami1-1* cross than in the control cross (data not shown).

The *mat*− *FMR1* and *SMR2* genes are required for proper recognition of *mat*+ and *mat*− nuclei during dicaryotic cell formation: in crosses between a *mat*+ tester strain and a strain bearing a mutation in *FMR1* or *SMR2* the progeny is diminished and produces mainly uninucleate croziers and uniparental asci containing markers of the *mat*− mutant partner (Zickler *et al.* 1995; Arnaise *et al.* 1997). The quantitative and qualitative characteristics of the progeny were similar when the relevant *mat*− mutants were crossed to either an *ami1-1* or an *ami1*$^+$ strain (data not shown).

Our second purpose was to perform crosses in which the *ami1-1* mutation was artificially homozygous. This situation can be obtained by the following procedure: strains carrying a deletion of the *mat* locus and the entire *mat*− information integrated at ectopic positions are available (Coppin *et al.* 1993), and such Δ*mat* (*mat*−) strains (in which the information between parentheses is transgenic) are fully functional and can be crossed to *mat*+ strains. Since the transgenic information segregates in crosses, some ascospores bear, in the same nucleus, the resident *mat*+ and the ectopic *mat*− information. Such *mat*+ (*mat*−) strains are able to self-fertilize, although they yield a very scant progeny. However, crosses between these dual maters and a *mat*− tester strain are fertile (as are classic *mat*+ × *mat*− crosses; E. Coppin and S. Arnaise, personal communication; see also Coppin *et al.* 1997). The *ami1-1 mat*+ strain was thus crossed to a Δ*mat* (*mat*−) strain and uninucleate *ami1-1 mat*+ ascospores carrying the *mat*− transgenic information were recovered. Their phenotypic properties were compared to those of *ami1*$^+$ *mat*+ (*mat*−) strains. As shown in Figure 3C, and in contrast to control strains, the *ami1-1 mat*+ (*mat*−) strains are self-sterile, even though they develop more protoperithecia than normal and are able to cross with both *mat*+ and *mat*− tester strains. Tests of their female capacity were performed according to the procedure described above and showed that the *ami1-1 mat* + (*mat*−) strains can act as female partners in crosses with both *mat*+ and *mat*− testers. Additional tests (as for *ami1-1*) provided evidence that their filaments act as male partners with

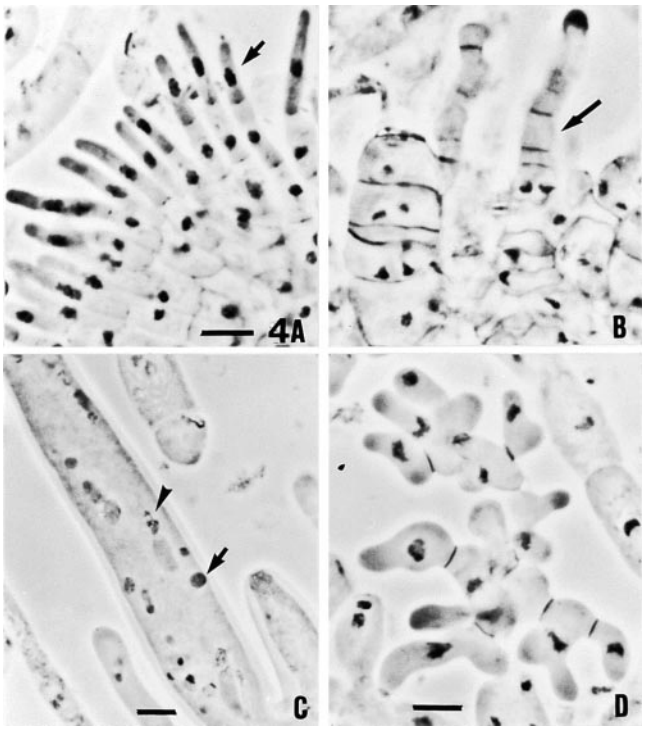

Figure 4.—The *ami1-1* mutant displays abnormal paraphysae, croziers, and asci. (A and B) Paraphysae from the perithecial beak. In wild-type perithecia (A), the different cells of these paraphysae are regular in shape and are mainly uninucleate (arrow). In *ami1-1* paraphysae (B), they are irregular in shape and contain either one (top left) or several nuclei (base cells); moreover, the terminal cells are mainly anucleate (large arrow). (C and D) *ami1-1 mat*+ (*mat*−) × *ami1*$^+$ *mat*−. (C) Abnormal ascus with small and anucleate ascospores (arrow); note also that nuclei (arrowhead) are excluded from the ascospores. (D) Each perithecium contains croziers that are uninucleate and abnormal in shape. Bar, 5 μm.

both testers (data not shown). Thus, the self-sterility of the *ami1-1 mat*+ (*mat*−) strain could be due to the fact that either fertilization *per se*, or an event immediately following fertilization, is prevented when the *ami1-1* mutation is present in both the male and female structures. In crosses between *ami1-1 mat*+ (*mat*−) used as the female partner and *mat*− strains, petri dishes contained, in addition to numerous protoperithecia, from 10 to 20 small and empty perithecia and a few larger ones in which only half the asci contained four ascospores. The other asci showed atypical ascospore formation due to abnormal distribution of the postmeiotic nuclei in the asci (Figure 4C), but earlier meiotic stages were normal. It is striking that, at the end of their development, these fruiting bodies also exhibit croziers that are uninucleate and sometimes abnormal in their shape and, consequently, in their development toward meiocytes (Figure 4D). As described above, abnormal asci were also observed in *ami1-1 mat*+ × *ami1*$^+$ *mat*− crosses. However, defects in crozier formation were seen only in the *ami1-1 mat*+ (*mat*−) × *ami1*$^+$ *mat*− crosses.

The *ami1* gene is the functional homologue of the *A. nidulans apsA* gene: The *ami1* gene appeared tightly linked to the *mat* locus: no recombination was observed in crosses between the wild-type *mat*− and *ami1-1 mat*+ strains among >100 uninucleate ascospores examined. Three cosmids encompassing the mating-type region (Figure 5) were used to transform an *ami1-1* recipient and all three complemented the mutant with respect to its male-sterile phenotype (data not shown). A precise localization of the gene on the N9 cosmid was obtained according to the procedure described by Turcq *et al.* (1990). Since a subclone containing the whole *ami1* gene was not obtained, it was sequenced using several subcloned fragments and the cosmid N9 (Figure 5).

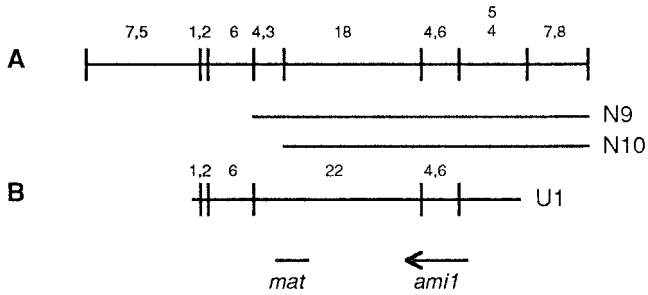

Figure 5.—*Eco*RI maps of the *mat* locus (A: *mat*−, B: *mat*+). The number above each fragment gives its size in kilobases. Superposed numbers indicate the size of unordered fragments. Cosmid inserts end with either *Eco*RI (N9 and N10) or *Sau*3A (U1) sites. The arrow shows the position and orientation of the *ami1* gene.

Analysis of the nucleotide sequence identifies an open reading frame of 1882 codons, interrupted by one putative intron. Sequence comparisons revealed a high similarity between the putative protein AMI1 and the *A. nidulans* ApsA protein (Fischer and Timberlake 1995). As shown in Figure 6, the *A. nidulans* and *P. anserina* proteins display 42% identity and 59% similarity in a 1676-amino-acid overlap. The cDNA was also completely sequenced and the presence of a single 56-bp intron (see asterisk in Figure 6) was confirmed; it is located at exactly the same position as the *apsA* intron.

All structural features reported for ApsA (Fischer and Timberlake 1995) are also found in AMI1. Both proteins are hydrophilic, with an overall pI of 5.2 for AMI1 (5.0 for ApsA). Three imperfect repeats of ~25 amino acids (for AMI1) are found in the central and most acidic region of the two proteins (residues 900–1400 in AMI1). In both cases, the N-terminal portions of the proteins show a high probability of coiled-coil formation, along with the presence of several heptad repeats (5 in AMI1 and 25 in ApsA). Both proteins contain a PH domain (Gibson *et al.* 1994 for a review) in their C-terminal parts (residues 1603–1715 in AMI1). In addition, in AMI1, this region (residues 1840–1880) also displays a high probability (>0.93) of coiled-coil formation (Lupas *et al.* 1991), a feature not exhibited by the C-terminal part of ApsA.

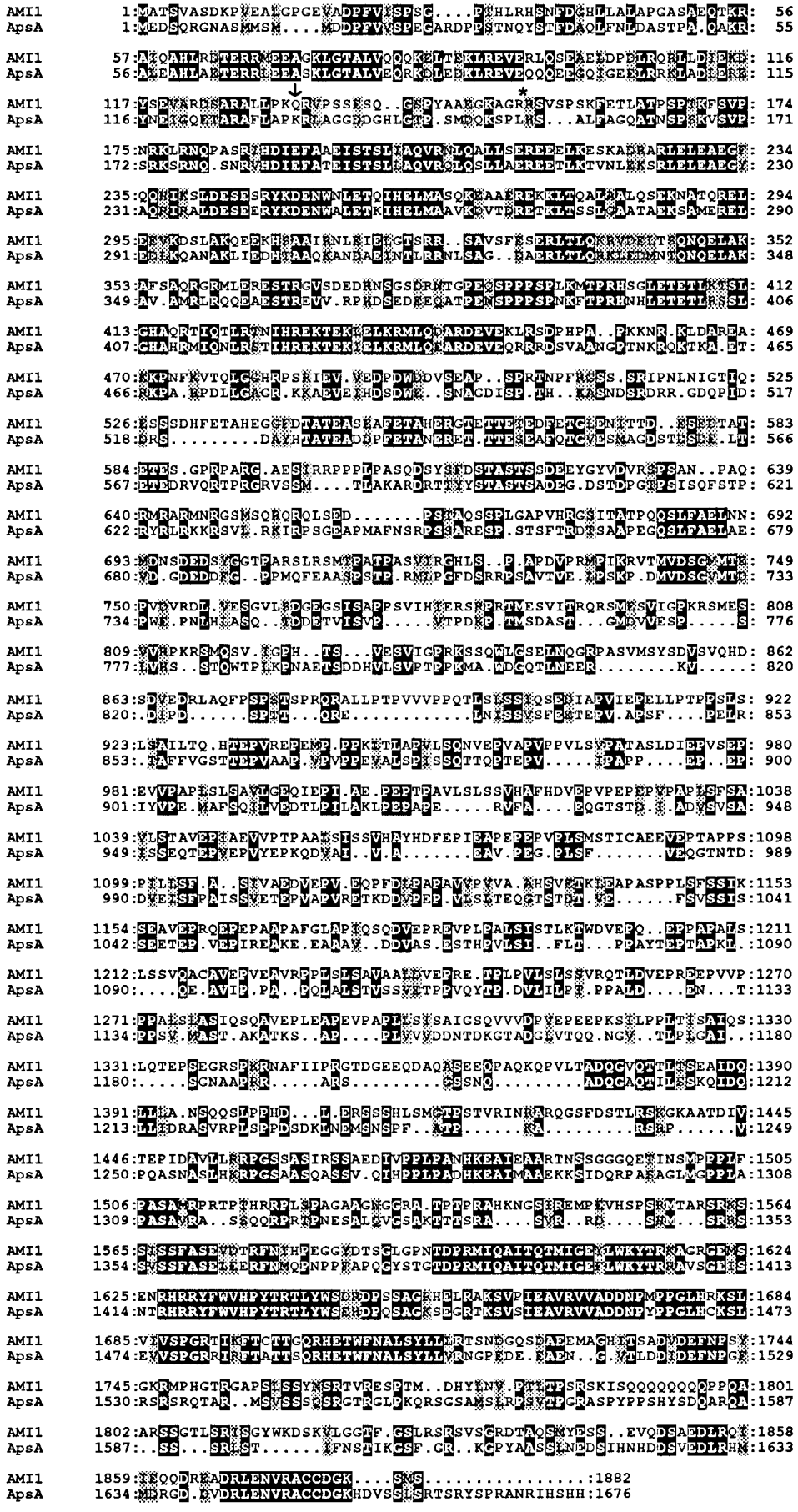

Figure 6.—Comparison of the amino acid sequences deduced from the *P. anserina ami1* gene (top line) and the *A. nidulans apsA* gene (bottom line). Similar and identical amino acids are shown, respectively, in gray and black boxes. The localization of the stop codon in the *ami1-1* mutant is designated by an arrow. The asterisk above the sequence shows the intron position. The alignment was obtained with Pileup algorithm, with a gap penality of 1.000 and a length penality of 0.100. The figure was obtained using the Boxshade program. The DDB/EMBL/GenBank accession number for the *ami1* nucleotide sequence is AJ271779 PAN271779.

Sequence comparison also revealed a significant similarity between AMI1 and *S. cerevisae* Num1p (Kormanec *et al.* 1991), as previously reported for ApsA (Fischer and Timberlake 1995). In both cases, similarity to the yeast protein (2748 residues) is limited to the C-terminal region of the proteins, including the PH domain (39% identity between AMI1 and Num1p in a 228-amino-acid overlap). However, structural similarities are observed between the three proteins: Num1p also displays heptad repeats in its N terminus, direct repeats in its central part, and a PH domain in its C terminus (Kormanec *et al.* 1991; Fischer and Timberlake 1995; this study).

Northern blot analyses, using the 4.6-*Eco*RI fragment (Figure 5) as a probe, revealed a 6.5-kb transcript whose abundance was similar in *mat*+ and *mat*− mycelia (data not shown).

Heterologous Southern hybridization experiments were performed using two probes corresponding to the 5′ and 3′ regions of *ami1* (materials and methods). In both cases we observed a clear signal with DNA from *A. nidulans* and an even stronger signal with DNA from *A. immersus*. There were no signals with the DNA from the other organisms tested, even after long exposure of the blot, despite positive controls with the eEF1A probe (data not shown).

The *ami1-1* mutant was obtained by a repeat-induced point mutation (RIP)-like mechanism. Until now, RIP has been described only in *N. crassa* (Selker *et al.* 1987; Selker 1997 for a review). The characteristics of the *P. anserina* RIP-like process will be described elsewhere (F. Graïa, O. Lespinet, R. Rimbault, E. Coppin, M. Dequard-Chablat and M. Picard, unpublished results). With respect to the present purpose, the first 2 kb of one of the two *ami1* copies present in the tandem duplication was sequenced after loss of the second copy by a recombination/excision (looping out) process. This revealed numerous G:C to A:T mutations, one of which created a nonsense codon 391 bp after the initiation codon (the localization of the mutation is designated by an arrow in Figure 6). Although the status of the second *ami1* copy remains unknown, the stringency of the *ami1-1* phenotype strongly suggests that the two copies are inactivated in the strain used in this study.

The clear similarity between the *P. anserina* AMI1 and the *A. nidulans* ApsA proteins strongly suggested that the two genes could be functional homologues. To test this hypothesis, the *apsA* gene (kindly provided by Dr. R. Fischer) was introduced in the *ami1-1* strain through cotransformation with a plasmid carrying a selectable marker, the bacterial hygromycin resistance gene (materials and methods). Among the hygromycin-resistant primary transformants, some exhibited a male-fertile phenotype. Two of these were purified through crosses with a *mat*− tester strain. They correspond to two transgenic strains carrying the *apsA* gene integrated in the *P. anserina* genome and called (*apsA-1*) and (*apsA-12*). As shown in Figure 1, one transformant, *ami1-1* (*apsA-1*), exhibited an efficient complementation of the mutant phenotype with respect to male fertility while the other, *ami1-1* (*apsA-12*), displayed partial complementation. Cytological observations showed that some microconidia were indeed nucleate but that their percentage was very low in both transformants: about 3–4% in *ami1-1 apsA-1* and <1% in *ami1-1 apsA-12*, compared to 0.1% in *ami1-1* strains. These results were confirmed by functional tests (Table 1). Interestingly, in spite of the poor complementation with respect to conidia nucleation, the *apsA-1* transgene decreases the amount of abnormal asci when present in crosses between *ami1-1* and the wild type: 20–25% [*ami1-1* (*apsA-1*) × *ami1*$^+$] *vs.* 36–38% (*ami1-1* × *ami1*$^+$). Moreover, it is able to restore self-fertility when present in *mat*+ (*mat*−) strains carrying the *ami1-1* mutation (Figure 3C). However, the perithecia issued from a cross between this strain and a *mat*− tester strain remain mostly barren, although in this case the *mat*− strain was used as the female partner: the *ami1-1* (*apsA-1*) *mat*+ (*mat*−) is male fertile and its self-fertility does not permit its use as a female partner in a cross. Cytological analyses did not show significant differences between the two crosses: *ami1-1 mat*+ (*mat*−) × *ami1*$^+$ *mat*− (see above) and *ami1-1 mat*+ (*mat*−) (*apsA-1*) × *ami1*$^+$*mat*−. These data lead to two conclusions: first, the *apsA* transgene does not complement this defect in the *P. anserina* mutant; second, this defect is not due to a maternal effect of the *ami1-1* mutation.

We also examined the possible effects of the *apsA-1* transgene in an *ami1*$^+$ background. The relevant transgenic strains did not exhibit any particular phenotype with respect to microconidial nucleation and sexual reproduction in crosses bearing the transgene in a homozygous or heterozygous state (data not shown).

DISCUSSION

Genetic control of nuclear migration events required for transition to the cellular state in filamentous ascomycetes: As emphasized in the Introduction, nuclear migration events are common to all eukaryotes; however, the genetic control of these processes is still incompletely understood, even in systems amenable to classical and molecular genetics. Nevertheless, systematic studies, especially those performed in filamentous ascomycetes (Fischer 1999 for a review), have designated two classes of genes implicated in these processes: those that are essential or limiting for cell survival and have pleiotropic roles in both vegetative growth and development, and those that function primarily at the level of developmental processes (see Introduction). We will focus here on the second class, because of the remarkable ability exhibited by filamentous ascomycetes to achieve syncytial/cellular transitions at several steps of their life cycle.

Many genes controlling conidiophore development have been characterized in *A. nidulans* (Adams *et al.*

1998 for a review). Among them, *apsA* and *apsB* (Clutterbuck 1994) are required at the transition between the syncytial state (which is maintained in the conidiophore stalk) and the initial uninucleate state corresponding to the metula cells (Adams *et al.* 1998). *apsA* and *apsB* mutants grow quite normally, while displaying abnormal nuclear distribution in the mycelial filaments. The most conspicuous phenotype of these mutants is their very poor efficiency in nuclear transmission to the metulae, such that these cells are mostly anucleate, blocking further development. Interestingly, when a metula receives a nucleus, conidia are formed as in the wild type. Thus, the *apsA* and *apsB* genes are required at a very precise stage of conidiophore development (Clutterbuck 1994; Fisher and Timberlake 1995). The main difference between *apsA* and *apsB* mutants concerns sexual reproduction, which also requires a transition from a syncytial to a cellular state (see below). While sexual development is quite normal in *apsA* mutants (Krüger and Fisher 1996) with the exception of one of the original mutants (which is sterile), all *apsB* mutants give low yields of ascospores (Clutterbuck 1994) but the step altered in these mutants remains unknown.

In *N. crassa*, orthologues of *apsA* and *apsB* have not yet been described (Springer 1993; Maheshwari 1999 for reviews). One can postulate that these genes are not involved in the macroconidiation pathway: macroconidia are plurinucleate and their formation does not require a loss of the syncytial state. In contrast, microconidia are uninucleate and their nucleation might require *apsA* and *apsB* orthologues.

In *P. anserina*, which is closely related to *N. crassa* and evolutionarily very distant from *A. nidulans*, a systematic search for genes involved in nuclear migration events has not yet been undertaken. However, in this species, several genes required for a proper transition between the syncytial and the cellular states during sexual reproduction have been characterized. This transition occurs when pairs of nuclei must migrate from the sexual plurinucleate cells into the ascogenous hyphae and croziers, to form the dicaryotic stage required for caryogamy, meiosis and ascospore formation (Zickler 1973). The *cro1* gene is required for this syncytial/cellular transition. Croziers formed in a *cro1-1* mutant are mainly plurinucleate (Simonet and Zickler 1978). We do not yet know if the role of the *cro1* gene is situated at the nuclear migration process necessary for exit from the plurinucleate state and/or if it is involved after this transition, to control the coordination between mitoses and cell divisions required for maintenance of the dicaryotic state. The *cro1-1* mutant grows slowly but nuclear distribution is normal in vegetative filaments (Berteaux-Lecellier *et al.* 1998). The mating-type genes are also required for a proper transition to the dicaryotic state because they ensure nuclear recognition between *mat*+ and *mat*− nuclei at this critical step of sexual reproduction. Cytological and genetic data have shown that inactivation of one of these genes results in croziers that are mostly uninucleate and to a diminished progeny, mainly uniparental, with the genotype of the *mat* mutant partner. Our hypothesis is that the loss of mating-type expression in the mutant nuclei disturbs nuclear migration such that ascogenous hyphae are mostly populated by the mutant nuclei (Zickler *et al.* 1995; Arnaise *et al.* 1997; Debuchy 1999). Interestingly, in both cases (as mentioned above for the *aps* mutations), the mutations do not preclude further development. Plurinucleate (*cro1-1*) and uninucleate (*mat* mutants) cells can undergo caryogamy, followed by polyploid (*cro1-1*) or haploid (*mat* mutants) meiosis, and, when meiosis does not abort, ascospores are produced (Zickler *et al.* 1995; Berteaux-Lecellier *et al.* 1998).

***ami1* is the functional orthologue of *apsA* but has broader functions:** Both genes encode proteins that display 42% identity and share all structural features previously reported for ApsA (Fisher and Timberlake 1995). Furthermore, *apsA* is able to complement, at least partially, some phenotypic defects of the *ami1-1* mutant. However, the *ami1-1* mutation is more pleiotropic than the *apsA* mutations and causes defects not only in conidiation, but also in sexual reproduction (summarized in Table 2).

Overall, the *ami1-1* defects can be grouped in three classes: abnormal distribution of nuclei in plurinucleate cells, lack of nuclear transmission at the syncytial/cellular transition, and inability to self-fertilize. The first type of defect occurs in vegetative filaments (as in *apsA* mutants) but also in part of the asci, thus hampering correct ascospore formation. In wild-type asci, nuclei that cooperate in ascospore formation are placed in close proximity by the arrangement of the PMM spindles: after division, each nuclear pair remains linked by astral microtubules and they must migrate before binucleate ascospore formation (Thompson-Coffe and Zickler 1992). Cortical, astral, and spindle microtubules as well as spindle pole bodies appear normal in an *ami1-1* × wild-type cross. Conidiophore microtubules and actin filaments were also found normal in the *apsA* mutant of *A. nidulans* (Fisher and Timberlake 1995). Thus, the role played by the ApsA/AMI1 protein in nuclear positioning remains unknown.

The second type of defect is observed during conidiation. This is also a property in common with *apsA* mutants, even though conidiation is far more complex in *A. nidulans* than in *P. anserina*. However, in the *ami1-1* mutant, this type of defect is also encountered at two other stages of the life cycle involving a syncytial/cellular transition: the formation of the perithecial beak paraphysae and of the croziers. In the first case, the mutant shows a maternal effect, since these structures are issued from the perithecial envelope whose origin is maternal. Instead of forming regularly uninucleate cells as in the wild type, the mutant maintains the plurinucleate state in one or two cells and then forms cells that are normally septate but anucleate and misshapen. In the second

TABLE 2

Summary of *ami1-1* phenotypic defects and complementation (no complementation) by *apsA-1*

Fungal structures or events	*ami1-1*	*ami1-1* (*apsA1*)
Mycelial filaments	Nuclei clustered	Nuclei clustered
Microconidia	0.1% nucleate	3% nucleate
Self-fertilization	Null[a]	Restored[a]
Beak paraphysae	Anucleate apical cells[b]	Partly restored
Croziers	Some uninucleate[c]	Some uninucleate
Postmeiotic nuclear distribution	Abnormal in 36–38% asci[d]	Abnormal in 20–25% asci[d]

[a] In strains carrying both mating types in the same nucleus: *ami1-1 mat+* (*mat−*) *vs. ami1-1* (*apsA-1*) *mat+* (*mat−*).
[b] Maternal effect.
[c] In crosses: *ami1-1 mat+* (*mat−*) × *ami1*$^+$ *mat−* *vs. ami1-1* (*apsA-1*) *mat+* (*mat−*) × *ami1*$^+$ *mat−*.
[d] In *ami1-1* or *ami1-1* (*apsA-1*) × *ami1*$^+$ crosses.

case, while wild-type croziers are always binucleate, *ami1-1* perithecia yield a few uninucleate and abnormal croziers. This was observed in a particular genetic background in which the *ami1-1* nucleus carries the two mating-type information. The reasons for which this context is more sensitive to the *ami1-1* mutation (while heterozygous) and why only some croziers are abnormal remain unclear.

The third type of *ami1-1* defect is the inability to self-fertilize. Since we could not perform *ami1-1 mat+* × *ami1-1 mat−* crosses, we took advantage of the previous observation that strains carrying the two *mat* information in the same (haploid) nucleus displayed selfing (Picard *et al.* 1991). An *ami1-1 mat+* (*mat−*) strain is unable to self-fertilize. This strain is able to act as a male partner (through filaments) in a cross and develops numerous protoperithecia (female organs) but only a few are available for fertilization by exogenous (wild-type) microconidia. Thus, the strain can act as a female partner, but to a limited extent. The simplest explanation of these observations is as follows: recognition between male and female structures indeed occurs in the *ami1-1 mat+* (*mat−*) strain but the *ami1-1* male nucleus is unable to migrate in the *ami1-1* female trichogyne, thus hampering perithecial development. This block occurs so early that fertilized protoperithecia cannot be distinguished from those that are unfertilized. However, the signal caused by this pseudofertilization event would render impossible a true fertilization of the relevant female structures by an exogenous male gamete. Notably, this selfing defect is complemented by *apsA*, which also increases the percentage of nucleate microconidia but seems unable to complement other defects of *ami1-1*. These differences in the complementation abilities of *apsA* will be discussed below.

***NUM1*/*apsA*/*ami1*: an example of the functional evolution of a gene:** Both ApsA (Fischer and Timberlake 1995) and AMI1 (this article) proteins show striking structural similarities with the protein encoded by the *NUM1* gene of *S. cerevisiae* (Kormanec *et al.* 1991), although primary sequence similarities are limited to the C-terminal portions. *num1* mutant strains grow normally but exhibit abnormal distribution of nuclei in budded cells, with two nuclei in the mother cell. In fact, in the mutant the order of the nuclear migration and nuclear division events is reversed compared to the wild type. Normally, nuclear migration to the bud neck occurs before mitosis, while in the mutant this occurs after nuclear division (Kormanec *et al.* 1991; Revardel and Aigle 1993). *NUM1* shows a cell-cycle-dependent expression and its product associates with the cortex of mother cells, but not with the bud. Genetic and cytological data suggest that the NUM1 protein controls nuclear migration by affecting microtubule functions (Farkasovsky and Küntzel 1995). The ApsA protein was also localized at the cytoplasmic membrane along the mycelial filaments and at the septa (Suelmann *et al.* 1997). In contrast, the *apsB* gene encodes a cytoplasmic protein. The remarkable point is that, in the *apsB* null mutant, nuclei show increased chaotic movements, which may explain their clustering in filaments (Suelmann *et al.* 1998). The data overall led the authors to propose an attractive hypothesis for the developmental defect of the mutants (anucleate metulae), rather than the simple hypothesis based on a nuclear migration failure: the nuclei would migrate into metulae but, because of an increased motility, would exit the cells before septum formation. Thus, the *aps* genes would regulate nuclear positioning and not nuclear movements *per se* (Suelmann *et al.* 1997, 1998).

The functional evolutionary relationship between *apsA* and *ami1* can be discussed at two (overlapping) levels: the partial complementation of *ami1-1* by *apsA* and the differences between phenotypic defects caused by null mutations in the two genes. Of the two defects shared by *apsA* and *ami1* mutants (summarized in Table 2, first two rows), one is partly rescued by the *apsA* transgene, which increases 30-fold the percentage of nucleate microconidia produced by *ami1-1*. However, this corresponds only to 3% of the wild-type level. This

partial complementation (and the lack of detectable complementation of abnormal distribution of nuclei in the mycelium) can be explained in two (nonexclusive) fashions. First, the *apsA* gene might be underexpressed, due either to position effects in the two integration events analyzed, or to an inefficient utilization of its promoter in *P. anserina*. In *A. nidulans*, ApsA is a low-abundance protein (Suelmann *et al.* 1997) and in *P. anserina*, it might be produced below the threshold required for full complementation of the *ami1-1* mutant. The second explanation for partial complementation relies upon the partners with which the ApsA protein must interact to be fully functional and that could be, at least partially, different in the two fungi. This second hypothesis is suggested by the differences in phenotypic defects displayed by the *apsA* and *ami1* mutants. The most striking difference is seen at the level of sexual reproduction: *ami1-1 mat+* (*mat−*) strains are unable to self-fertilize while sexual reproduction of *apsA* null mutants is quite normal (Krüger and Fischer 1996). Since *P. anserina* and *A. nidulans* are evolutionarily distant, the two genes may have evolved such that *ami1* would play a general role, while the role of *apsA* would be restricted to the asexual part of the life cycle. However, several puzzling observations suggest that *apsA* might play some role in sexual reproduction. First, a mutant allele of *apsA* causes sterility (Clutterbuck 1994) but its precise defect is not known. Second, among the two analyzed extragenic suppressors of *apsA*, one (*samA*$^-$) fails to produce fruiting bodies (cleistothecia) and the other (*samB*$^-$) gives barren cleistothecia (Krüger and Fischer 1996). Deletion of the *samB* gene has shown that it is involved in morphogenesis rather than in nuclear distribution *per se*: the mutation causes premature hyphal ramifications and mislocalization of septa. Thus, its suppression effect upon *apsA* mutants may be very indirect (Krüger and Fischer 1998). However, its sexual defect has not been investigated precisely. The third and most amazing point concerning *apsA* and sexual reproduction is its ability to restore self-fertilization of *ami1-1 mat+* (*mat−*) strains. There is, in fact, an impressive parallel between *apsA*/*ami1* and the *rca1*/*flbD* genes. *flbD* is an *A. nidulans* gene that encodes a DNA-binding protein involved in initiation of conidiophore development (Adams *et al.* 1998 for a review). *rca1* is a *N. crassa* gene that seems to be the functional orthologue of *flbD* since it fully complements the *A. nidulans flbD* mutant. The striking point is that deletion of *rca1* in *N. crassa* does not cause any major effect on conidiation and sexual reproduction (Shen *et al.* 1998). As the authors conclude, if *rca1* is involved in *N. crassa* development, its role is subtle or redundant. This conclusion might also be applied to *apsA* with respect to sexual reproduction. Interestingly, the *A. nidulans flbD* gene was the sole among six regulators of conidiation that crosshybridized with *N. crassa* DNA (Shen *et al.* 1998).

This heterologous hybridization approach has been used with two fragments of the *ami1* gene. There is no signal with *S. macrospora*, which is closely related to *P. anserina*, while there is a clear-cut signal with *A. nidulans* and *A. immersus*, which are very distant from each other and from *P. anserina*. *S. macrospora* and *P. anserina* belong to the same family (Sordariacae) but *S. macrospora* is aconidial and homothallic. This does not explain, *per se*, the lack of hybridization since sexual development is similar in these two fungi. *S. macrospora* may, however, have an orthologue of *apsA*/*ami1*, which might have evolved significantly at the nucleotidic sequence level. *A. nidulans* and *A. immersus* belong to the two other subgroups of filamentous ascomycetes: Plectomycetes and Discomycetes, respectively. *A. nidulans* is homothallic and differentiates conidia, while *A. immersus* is heterothallic and aconidial. It would be of great interest to clone the *A. immersus apsA*/*ami1* orthologue to learn more about the functional evolution of these genes. It would also be interesting to identify a possible orthologue in Homobasidiomycetes and understand its putative role in nuclear migration at mating. In these organisms, the process is controlled by a pheromone/receptor control via one of the two mating-type loci (Wendland *et al.* 1995; Casselton and Olesnicky 1998 for a review). However, the genes acting downstream of this signal remain to be discovered.

The major role of *apsA*/*ami1* is to regulate nuclear movements and/or positioning; these genes are thus essential for proper development at one or several steps that require a syncytial/cellular state transition. It is of the utmost importance, in terms of the functional evolution of this class of genes, to discover if they have orthologues in multicellular organisms, especially those that display syncytial/cellular transitions in their development. This concerns not only insect embryogenesis (see Schejter and Wieschaus 1993 for a review on Drosophila) but also those plants which require this type of transition for formation of their female gametes (Huang and Sheridan 1994 and references therein) or in their early embryonic development (Johansen 1950).

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LITERATURE CITED

Adams, T. H., J. K. Wieser and J. H. Yu, 1998 Asexual sporulation in *Aspergillus nidulans*. Microbiol. Mol. Biol. Rev. **62:** 35–54.

Arnaise, S., R. Debuchy and M. Picard, 1997 What is a *bona fide*

mating-type gene? Internuclear complementation of *mat* mutants in *Podospora anserina*. Mol. Gen. Genet. **256:** 169–178.

Barth, P. G., 1987 Disorders of neuronal migration. Can. J. Neurol. Sci. **14:** 1–16.

Beckwith, S. M., C. H. Roghi, B. Liu and N. R. Morris, 1998 The "8-kD" cytoplasmic dynein light chain is required for nuclear migration and for dynein heavy chain localization in *Aspergillus nidulans*. J. Cell Biol. **143:** 1239–1247.

Bernet, J., 1965 Mode d'action des gènes de "barrage" et relation entre l'incompatibilité cellulaire et l'incompatibilité sexuelle chez *Podospora anserina*. Ann. Sci. Nat. Bot. **6:** 611–768.

Bernet, J., 1986 *Podospora* mutations reducing cell survival following nutrient exhaustion. Curr. Genet. **14:** 133–136.

Bernet, J., 1988 *Podospora* growth control mutations inhibit apical cell anastomosis and protoperithecium formation. Exp. Mycol. **12:** 217–222.

Berteaux-Lecellier, V., M. Picard, C. Thompson-Coffe, D. Zickler, A. Panvier-Adoutte *et al.*, 1995 A nonmammalian homolog of the *PAF1* gene (Zellweger syndrome) discovered as a gene involved in caryogamy in the fungus *Podospora anserina*. Cell **81:** 1043–1051.

Berteaux-Lecellier, V., D. Zickler, R. Debuchy, A. Panvier-Adoutte, C. Thompson-Coffe *et al.*, 1998 A homologue of the yeast *SHE4* gene is essential for the transition between the syncytial and cellular stages during sexual reproduction of the fungus *Podospora anserina*. EMBO J. **17:** 1248–1258.

Camonis, J. H., M. Cassan and J. P. Rousset, 1990 Of mice and yeast: versatile vectors which permit gene expression in both budding yeast and higher eukaryotic cells. Gene **86:** 263–268.

Casselton, L. A., and N. S. Olesnicky, 1998 Molecular genetics of mating recognition in basidiomycete fungi. Microbiol. Mol. Biol. Rev. **62:** 55–70.

Church, G. M., and W. Gilbert, 1984 Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:** 1991–1995.

Clutterbuck, A. J., 1994 Mutants of *Aspergillus nidulans* deficient in nuclear migration during hyphal growth and conidiation. Microbiology **140:** 1169–1174.

Coppin, E., S. Arnaise, V. Contamine and M. Picard, 1993 Deletion of the mating-type sequences in *Podospora anserina* abolishes mating without affecting vegetative functions and sexual differentiation. Mol. Gen. Genet. **241:** 409–414.

Coppin, E., R. Debuchy, S. Arnaise and M. Picard, 1997 Mating types and sexual development in filamentous ascomycetes. Microbiol. Mol. Biol. Rev. **61:** 1092–2172.

Cunniff, J., Y. H. Chiu, N. R. Morris and R. Warrior, 1997 Characterization of *DnudC*, the *Drosophila* homolog of an *Aspergillus* gene that functions in nuclear motility. Mech. Dev. **66:** 55–68.

Debuchy, R., 1999 Internuclear recognition: a possible connection between Euascomycetes and Homobasidiomycetes. Fungal Genet. Biol. **27:** 218–223.

Debuchy, R., and Y. Brygoo, 1985 Cloning of opal suppressor tRNA genes of a filamentous fungus reveals two $tRNA^{ser}$ UGA genes with unexpected structural differences. EMBO J. **4:** 3553–3556.

Deleu, C., C. Clave and J. Begueret, 1993 A single amino acid difference is sufficient to elicit vegetative incompatibility in the fungus *Podospora anserina*. Genetics **135:** 45–52.

Esser, K., 1974 *Podospora anserina*, pp. 531–551 in *Handbook of Genetics*, Vol. 1, edited by R. C. King. Plenum, New York.

Farkasovsky, M., and H. Küntzel, 1995 Yeast Num1p associates with the mother cell cortex during S/G2 phase and affects microtubular functions J. Cell Biol. **131:** 1003–1014.

Fischer, R., 1999 Nuclear movement in filamentous fungi. FEMS Microbiol. Rev. **23:** 39–68.

Fischer, R., and W. E. Timberlake, 1995 *Aspergillus nidulans apsA* (anucleate primary sterigmata) encodes a coiled-coil protein required for nuclear positioning and completion of asexual development. J. Cell Biol. **128:** 485–498.

Gibson, T. J., M. Hyvönen, A. Musacchio, M. Saraste and E. Birney, 1994 PH domain: the first anniversary. Trends Biochem. Sci. **19:** 349–353.

Hanahan, D., 1983 Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:** 557–580.

Huang, B. Q., and W. F. Sheridan, 1994 Female gametophyte development in maize: microtubular organization and embryo sac polarity. Plant Cell **6:** 845–861.

Jamet-Vierny, C., V. Contamine, J. Boulay, D. Zickler and M. Picard, 1997 Mutations in genes encoding the mitochondrial outer membrane proteins Tom70 and Mdm10 of *Podospora anserina* modify the spectrum of mitochondrial DNA rearrangements associated with cellular death. Mol. Cell. Biol. **17:** 6359–6366.

Johansen, D. A., 1950 *Plant Embryology*. Chronica Botanica Co., Waltham, MA.

Kormanec, J., I. Schaaff-Gerstenschläger, F. K. Zimmermann, D. Perecko and H. Küntzel, 1991 Nuclear migration in *Saccharomyces cerevisiae* is controlled by the highly repetitive 313 kDa NUM1 protein. Mol. Gen. Genet. **230:** 277–287.

Krüger, M., and R. Fischer, 1996 Isolation of two *apsA* suppressor strains in *Aspergillus nidulans*. Genetics **144:** 533–540.

Krüger, M., and R. Fischer, 1998 Integrity of a Zn finger-like domain in SamB is crucial for morphogenesis in ascomycetous fungi. EMBO J. **17:** 204–214.

Lecellier, G., and P. Silar, 1994 Rapid methods for nucleic acids extraction from Petri dish-growth mycelia. Curr. Genet. **25:** 122–123.

Lockington, R. A., C. Scazzocchio, D. Sequeval, M. Mathieu and B. Felenbock, 1987 Regulation of *alcR*, the positive regulatory gene of the ethanol utilization regulon of *Aspergillus nidulans*. Mol. Microbiol. **1:** 275–281.

Lu, B. C., 1967 The course of meiosis and centriole behaviour during the ascus development of the ascomycete *Gelasinospora calospora*. Chromosoma **22:** 210–226.

Lupas, A., M. van Dyke and J. Stock, 1991 Predicted coiled coils from protein sequences. Science **252:** 1162–1164.

Maheshwari, R., 1999 Microconidia of *Neurospora crassa*. Fungal Genet. Biol. **26:** 1–18.

McConnell, S. J., L. C. Stewart, A. Talin and M. P. Yaffe, 1990 Temperature-sensitive yeast mutants defective in mitochondrial inheritance. J. Cell Biol. **111:** 967–976.

Minke, P. F., I. H. Lee, J. H. Tinsley, K. S. Bruno and M. Plamann, 1999 *Neurospora crassa ro-10* and *ro-11* genes encode novel proteins required for nuclear distribution. Mol. Microbiol. **32:** 1065–1076.

Morris, N. R., X. Xiang and S. M. Beckwith, 1995 Nuclear migration advances in fungi. Trends Cell Biol. **5:** 278–282.

Morris, S. M., P. Anaya, X. Xiang, N. R. Morris, G. S. May *et al.*, 1997 A prolactin-inducible T cell gene product is structurally similar to the *Aspergillus nidulans* nuclear movement protein NUDC. Mol. Endocrinol. **11:** 229–236.

Morris, S. M., A. Albrecht, O. Reiner, G. Eichele and L-Y. Yu-Lee, 1998 The lissencephaly gene product Lis1, a protein involved in neuronal migration, interacts with a nuclear movement protein, NudC. Curr. Biol. **8:** 603–606.

Orbach, M. J., 1994 A cosmid with a Hy^R marker for fungal library constructions and screening. Gene **150:** 159–162.

Osmani, A. H., S. A. Osmani and N. R. Morris, 1990 The molecular cloning and identification of a gene product specifically required for nuclear movement in *Aspergillus nidulans*. J. Cell Biol. **111:** 543–551.

Picard, M., 1971 Genetic evidences for a polycistronic unit of transcription in the complex locus "14" in *Podospora anserina*. I. Genetic and complementation maps. Mol. Gen. Genet. **111:** 35–50.

Picard, M., R. Debuchy and E. Coppin, 1991 Cloning the mating types of the heterothallic fungus *Podospora anserina*: developmental features of haploid transformants carrying both mating types. Genetics **128:** 539–547.

Plamann, M., P. F. Minke, J. H. Tinsley and K. S. Bruno, 1994 Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. J. Cell Biol. **127:** 139–149.

Prillinger, H., and K. Esser, 1977 The phenoloxidases of the ascomycete *Podospora anserina*: action and interaction of genes controlling the formation of laccase. Mol. Gen. Genet. **156:** 333–345.

Reiner, O., R. Carrozzo, Y. Shen, M. Wehnert, F. Faustinella *et al.*, 1993 Isolation of a Miller-Dieker lissencephaly gene containing G protein β-subunit-like repeats. Nature **364:** 717–721.

Revardel, E., and M. Aigle, 1993 The *NUM1* yeast gene: length polymorphism and physiological aspects of mutant phenotype. Yeast **9:** 495–506.

Rizet, G., 1952 Les phénomènes de barrage chez *Podospora anserina*. I. Analyse génétique des barrages entre souche S et s. Rev. Cytol. Biol. Veg. **13:** 51–92.

Rizet, G., and C. Engelmann, 1949 Contribution à l'étude gé-

nétique d'un ascomycète tétrasporé: *Podospora anserina* (Ces) Rehm. Rev. Cytol. Biol. Veg. **11:** 201–304.

Robb, M. J., M. A. Wilson and P. J. Vierula, 1995 A fungal actin-related protein involved in nuclear migration. Mol. Gen. Genet. **247:** 583–590.

Rosenthal, A., O. Coutelle and M. Craxton, 1993 Large-scale production of DNA sequencing templates by microtitre format PCR. Nucleic Acids Res. **21:** 173–174.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schejter, E. D., and E. Wieschaus, 1993 Functional elements of the cytoskeleton in early *Drosophila* embryo. Annu. Rev. Cell Biol. **9:** 67–99.

Selker, E. U., 1997 Epigenetic phenomena in filamentous fungi: useful paradigms or repeat induced confusion? Trends Genet. **13:** 296–301.

Selker, E. U., E. B. Cambareri, B. C. Jensen and K. R. Haack, 1987 Rearrangement of duplicated DNA in specialized cells of *Neurospora.* Cell **51:** 741–752.

Shen, W. C., J. Wieser, T. H. Adams and D. J. Ebbole, 1998 The *Neurospora rca-1* gene complements an aspergillus *flbD* sporulation mutant but has no identifiable role in *Neurospora* sporulation. Genetics **148:** 1031–1041.

Silar, P., and M. Picard, 1994 Increased longevity of EF-lα high-fidelity mutants in *Podospora anserina.* J. Mol. Biol. **235:** 231–236.

Simonet, J. M., and D. Zickler, 1978 Genes involved in caryogamy and meiosis in *Podospora anserina.* Mol. Gen. Genet. **162:** 237–242.

Springer, M. L., 1993 Genetic control of fungal differentiation: the three sporulation pathways of *Neurospora crassa.* Bioessays **15:** 365–374.

Suelmann, R., N. Sievers and R. Fischer, 1997 Nuclear traffic in fungal hyphae: *in vivo* study of nuclear migration and positioning in *Aspergillus nidulans.* Mol. Microbiol. **25:** 757–769.

Suelmann, R., N. Sievers, D. Galetzka, L. Robertson, W. E. Timberlake *et al.*, 1998 Increased nuclear traffic chaos in hyphae of *Aspergillus nidulans*: molecular characterization of *apsB* and *in vivo* observation of nuclear behaviour. Mol. Microbiol. **30:** 831–842.

Tarcsa, E., L. N. Marekov, J. Andreoli, W. W. Idler, E. Candi *et al.*, 1997 The fate of trichohyalin. Sequential post-translational modifications by peptidyl-arginine deiminase and transglutaminases. J. Biol. Chem. **272:** 27893–27901.

Thompson-Coffe, C., and D. Zickler, 1992 Three microtubule-organizing centers are required for ascus growth and sporulation in the fungus *Sordaria macrospora.* Cell Motil. Cytoskeleton **22:** 257–273.

Tinsley, J. H., P. F. Minke, K. S. Bruno and M. Plamann, 1996 $p150^{Glued}$, the largest subunit of the dynactin complex, is nonessential in *Neurospora* but required for nuclear distribution. Mol. Biol. Cell **7:** 731–742.

Turcq, B., M. Denayrolles and J. Begueret, 1990 Isolation of the two allelic incompatibility genes *s* and *S* of the fungus *Podospora anserina.* Curr. Genet. **17:** 297–303.

Vierula, P. J., and J. M. Mais, 1997 A gene required for nuclear migration in *Neurospora crassa* codes for a protein with cysteine-rich, LIM/RING-like domains. Mol. Microbiol. **24:** 331–340.

Wendland, J., L. J. Vaillancourt, J. Hegner, K. B. Lengeler, K. J. Laddison *et al.*, 1995 The mating-type locus *Bα1* of *Schizophyllum commune* contains a pheromone receptor gene and putative pheromone genes. EMBO J. **14:** 5271–5278.

Xiang, X., S. M. Beckwith and N. R. Morris, 1994 Cytoplasmic dynein is involved in nuclear migration in *Aspergillus nidulans.* Proc. Natl. Acad. Sci. USA **91:** 2100–2104.

Xiang, X., A. H. Osmani, S. A. Osmani, M. Xin and N. R. Morris, 1995 *NudF*, a nuclear migration gene in *Aspergillus nidulans*, is similar to the human *LIS-1* gene required for neuronal migration. Mol. Biol. Cell **6:** 297–310.

Zickler, D., 1973 La méiose et les mitoses au cours du cycle de quelques ascomycètes. Thèse d'Etat, Université Paris-Sud.

Zickler, D., S. Arnaise, E. Coppin, R. Debuchy and M. Picard, 1995 Altered mating-type identity in the fungus *Podospora anserina* leads to selfish nuclei, uniparental progeny, and haploid meiosis. Genetics **140:** 493–503.

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