The Podospora *rmp1* Gene Implicated in Nucleus-Mitochondria Cross-Talk Encodes an Essential Protein Whose Subcellular Location Is Developmentally Regulated

Véronique Contamine, Denise Zickler and Marguerite Picard[1]

Institut de Génétique et Microbiologie, Université Paris-Sud, UMR 8621, Orsay, France

Manuscript received June 26, 2003
Accepted for publication October 3, 2003

ABSTRACT

It has been previously reported that, at the time of death, the *Podospora anserina AS1-4* mutant strains accumulate specific deleted forms of the mitochondrial genome and that their life spans depend on two natural alleles (variants) of the *rmp1* gene: *AS1-4 rmp1-2* strains exhibit life spans strikingly longer than those of *AS1-4 rmp1-1*. Here, we show that *rmp1* is an essential gene. *In silico* analyses of eight *rmp1* natural alleles present in Podospora isolates and of the putative homologs of this orphan gene in other filamentous fungi suggest that *rmp1* evolves rapidly. The RMP1 protein is localized in the mitochondrial and/or the cytosolic compartment, depending on cell type and developmental stage. Strains producing RMP1 without its mitochondrial targeting peptide are viable but exhibit vegetative and sexual defects.

THE number of mitochondrial proteins has been estimated at 700–1000 (Schatz 1995; Wallace 1999; Kumar *et al.* 2002). The vast majority are encoded by nuclear genes and synthesized in the cytosol. Studies have focused on these "nuclear-mitochondrial genes" (Chinnery 2003) not only because of their fundamental interest in understanding the biogenesis and function of the organelle, but also in applied research to address the molecular bases of human diseases due to mitochondrial dysfunctions and mutations in nuclear genes. The yeast *Saccharomyces cerevisiae* has been a powerful model for these studies (*e.g.*, reviewed in Foury and Kucej 2001; Chinnery 2003). However, *S. cerevisiae* exhibits intrinsic weaknesses in this difficult path: it is a facultative aerobe and is unicellular. Thus, it is not surprising that some human nuclear-mitochondrial genes have no yeast homologs (reviewed in Foury and Kucej 2001; Chinnery 2003). Filamentous fungi, which are both strict aerobes and multicellular organisms, appear to be good complementary models. The genome sequences of several of these fungi are now available and it has been stressed that the number of human genes with homologs in the entire fungal kingdom is double that found with *S. cerevisiae* alone (Zeng *et al.* 2001).

In the filamentous ascomycete *Podospora anserina*, we have been interested in a degenerative process linked to the accumulation of mitochondrial genomes carrying specific deletions (mtDNA deletions; Belcour *et al.* 1991; reviewed in Belcour *et al.* 1999). These deleted mtDNA molecules accumulate only in strains bearing mutations in the nuclear *AS1* gene, which encodes a cytosolic ribosomal protein. Consequently, the effect of these mutations (*e.g.*, *AS1-4*) is indirect (Dequard-Chablat and Sellem 1994). This process exhibits similarities with human diseases characterized by the accumulation of mtDNA deletions. While some of these diseases are sporadic, others are inherited in a Mendelian fashion, thus implicating mutations in nuclear genes (reviewed in Larsson and Clayton 1995). Four genes for these disorders have been characterized, and all are nuclear-mitochondrial genes. They encode a thymidine phosphorylase (Nishino *et al.* 1999), an adenine nucleotide translocator (Kaukonen *et al.* 2000), a putative helicase (Spelbrink *et al.* 2001), and the mtDNA polymerase gamma (Goethem *et al.* 2001). In *P. anserina*, our aim has been to seek genes whose mutations or gene-dosage modifications can delay (or even abolish) the accumulation of the specific deleted mtDNA molecules and consequently increase the life span of the relevant *AS1-4* strains (Contamine and Picard 1998; Dequard-Chablat and Alland 2002). To date, four genes have been characterized, all of which are nuclear-mitochondrial genes. It is noteworthy that one, *pol G*, encodes the mtDNA polymerase gamma (M. Dequard-Chablat, personal communication). Another is *mthmg1* (Dequard-Chablat and Alland 2002), encoding a HMG-like protein, which is probably the homolog of the human transcription factor mtTFA/mtTF1 (Parisi and Clayton 1991). Interestingly, this factor is necessary for mtDNA maintenance in mice (Larsson *et al.* 1998). The two final genes, identified by our screening procedures in *P. anserina*, encode outer mitochondrial membrane proteins (Jamet-Vierny *et al.* 1997). These include TOM70, a well-conserved component of the receptor for protein import into mitochondria (Pfanner *et al.* 1996), as well

[1]*Corresponding author:* IGM, Bât. 400, Université Paris-Sud, 91405 Orsay Cedex, France. E-mail: picard@igmors.u-psud.fr

as MDM10, a protein previously identified in *S. cerevisiae* and involved in mitochondrial morphology and distribution (Sogo and Yaffe 1994).

Another gene, *rmp1*, was initially identified in *P. anserina* due to the presence of two natural alleles in our reference strains: *rmp1-1* (formerly *rmp*−) was found in the strain bearing the mating-type *mat*− information, while *rmp1-2* (formerly *rmp*+) was found in the *mat*+ strain. Both *AS1-4 rmp1-1* and *AS1-4 rmp1-2* strains accumulate the specific deleted mtDNA molecules at the time of death, but are strikingly different in their life spans, which are ~2 and 80 cm, respectively (Contamine *et al.* 1996). Here, we describe the cloning and analysis of *rmp1* by a multidisciplinary approach: searching for homologs in databases and construction of new alleles and expression studies throughout the life cycle. The results presented below demonstrate that *rmp1* is an essential nuclear-mitochondrial gene, which probably evolves rapidly and exhibits a complex expression pattern strictly regulated during development.

MATERIALS AND METHODS

***P. anserina* strains, growth conditions, and transformation:** *P. anserina* is a heterothallic filamentous ascomycete whose life cycle and general methods for genetic analysis have been described (Rizet and Engelmann 1949). The asci contain four binucleate spores, each formed around two nonsister nuclei after a postmeiotic mitosis. A few asci contain five spores, two of which are smaller and uninucleate. They give rise to homocaryotic mycelia, used mainly for genetic analyses. These strains develop both female organs (ascogonia) and male gametes (microconidia), but are unable to self-fertilize. The binucleate ascospores are used as tools, especially when heterocaryotic strains are required for complementation analyses. Crosses were performed by spermatization: a suspension of microconidia obtained from one strain (the male culture) was poured onto a homocaryotic mycelium (the female culture) of opposite mating type. Self-fertilization of heterocaryotic *mat*+/*mat*− strains was also examined. Cultures were usually performed at 27°. All media, *i.e.*, corn-meal extract (MR), minimal synthetic (M2), and germination (G) media were as described (Esser 1974). When required, G medium was supplemented with 1% yeast extract. M1 is the protoplast regeneration medium. When necessary, hygromycin (Roche Diagnostics), phleomycin (Sigma, St. Louis), or leucine were added to M1 at a concentration of 100, 5, and 100 μg/ml, respectively. Life spans were measured on M2 according to Contamine *et al.* (1996). Protoplast preparation and transformation experiments were performed as described previously (Berteaux-Lecellier *et al.* 1998).

The *rmp1* gene is tightly linked to the *mat* locus. The *rmp1-1* and *rmp1-2* (formerly *rmp*− and *rmp*+, respectively) are linked to *mat*− and *mat*+, respectively. Previous recombination data demonstrated that the genetic distance between *mat* and *rmp1* was ~0.25 cM (Contamine *et al.* 1996). Thus, *mat* can be used as a reliable marker of *rmp1*. All mutant strains are derived from the S strain (Rizet 1952). The *leu1-1* mutant is auxotrophic for leucine. The *AS1-4* mutation was identified as an antisuppressor mutation (Picard-Bennoun 1976). The origin and main features of the *P. anserina* wild-type isolates s and A and of the *P. comata* species were previously described (Contamine *et al.* 1996 and references therein).

Bacterial strains, cosmids, plasmids, and plasmid constructions: Cosmid and plasmid preparations were performed in either *Escherichia coli* DH5α (Hanahan 1983) or CM5α (Camonis *et al.* 1990).

The genomic library used in this study was constructed from a *rgs12 rmp1-1* strain (Dequard-Chablat and Alland 2002) in the integrative cosmid vector pMOcosX, with the bacterial hygromycin resistance gene under the control of the *cpc1* promoter of *Neurospora crassa* as selectable marker (Orbach 1994). The integrative plasmids used for constructions are derived from pCB1004 (Carroll *et al.* 1994) or pPaBle (Coppin and Debuchy 2000), which carry hygromycin or phleomycin resistance genes, respectively (Table 1). The pHSS and pPMB plasmids (Table 1) contain genomic fragments of 5.2 and 3.4 kb, respectively, both of which encompass the *rmp1-1* allele (Figure 1).

To obtain a frameshift mutation in *rmp1-1*, the pHSS plasmid was digested by *Nhe*I (Figure 1), successively followed by Klenow and ligation treatments. We thus obtained a +1 frameshift mutation and a stop codon between the two ATGs of the open reading frame (ORF) in position 138 (Figure 2), which yielded pHfs (Table 1). The modification in pHfs was confirmed by sequencing with primer 17 (Figure 1).

The pLSS plasmid (Table 1), used to obtain a null allele of *rmp1*, was constructed from pHSS, whose hygromycin resistance gene was deleted. The *rmp1-1* coding sequence was excised between the *Nhe*I site and the 3′ terminal *Nde*I site (Figure 1) and replaced by a *Xba*I-*Hin*dIII fragment carrying the *leu1* gene prepared from the pUL plasmid (Debuchy *et al.* 1993). A purified *Nsi*I-*Dra*III fragment (Figure 1) from pLSS was used for transformation-mediated gene replacement.

Constructions of the pHG, pHN, pHNG plasmids were achieved as follows. Two fragments were obtained by amplification from a bacterial artificial chromosome (BAC) containing the *rmp1-2* allele (provided by A. Billault, R. Debuchy, and P. Silar). The first was amplified with primers 21 and 17, followed by digestion with *Bam*HI and *Spe*I (Figure 1) and cloning into pHSS* (Table 1) digested by the same enzymes. This procedure gave rise to pHG. The second fragment was amplified with primers 8 and 11, followed by enzymatic digestion with *Cla*I and *Bsrg*I (Figure 1). The resultant product was cloned into pHSS* digested by *Bsrg*I and partially digested by *Cla*I (which has only one site in *rmp1-1*), giving rise to pHN. pHNG was constructed from pHN digested by *Bam*H1 and *Spe*I, followed by ligation with the initial PCR fragment digested by the same enzymes. The constructions were confirmed by sequencing, using primers 17 and 18 for pHG and pHNG and primer 11 for pHN (Figure 1).

The *rmp1-1::GFP* gene fusion was obtained as follows. A fragment of pHSS* (Table 1) was amplified with primers 8 (Figure 1) and Rgfp (5′-GAGGGTACCTCGCGATTACCGCG GAAGTTTTTCCCCGGCCCCCACCCAGT-3′). In this PCR product, the TAA stop codon of *rmp1-1* is replaced by CTT (leu), which is immediately followed by three restriction sites (*Sac*II, *Nru*I, and *Acc*65I). An in-frame TAA is present between *Sac*II and *Nru*I. In an initial step, the PCR fragment was digested by *Cla*I and *Acc*65I and cloned into pHSS* (Table 1), digested by *Bsrg*I and partially digested by *Cla*I (Figure 1). This construction was sequenced using primer 11 (Figure 1). We also verified that this modified form of *rmp1-1* retained the ability to complement the absence of aerial hyphae at 37° of a *rmp1-2* strain. Second, the pEGFP-1 plasmid (CLONTECH, Palo Alto, CA) was digested by *Cla*I and *Sac*II. The restriction fragment containing EGFP was ligated to the previous construction digested by *Sac*II and *Nru*I, giving rise to pHRGFP (Table 1).

pHRGFP was used to construct pHNRGFP, which contains the *rmp1-1::GFP* fusion deleted for 21 codons in the 5′ region

of the ORF (Δ3-23). A pHSS* fragment was amplified (Table1) using primers 25 (Figure 1) and Nrmp (5′-TGCACTGCAGT GAGCATTTGATTTGGTGCTTTCCT-3′). The PCR product was digested by *Mlu*I and *Pst*I (Figure 1) and cloned in pHRGFP digested by the same enzymes. This construction, bearing the *rmp1-1Δ(3-23)::GFP* allele, was sequenced using primers 19 and 25 (Figure 1). In addition to the deletion Δ(3-23), this form of *rmp1-1* also replaces the ala 24 codon with a threonine codon.

Isolation of strains bearing the Δ*rmp1* allele: The gene-replacement experiment yielding a genome bearing the Δ*rmp1* allele was performed by a strategy that permitted us to obtain the relevant transformants without phenotypic screening, even if the inactivation of *rmp1* were lethal. A transgenic *rmp1-1* strain (SS2, Table 2), carrying an ectopic functional copy of *rmp1-1* (carried by pHSS) was crossed with a *leu1-1* strain and a *leu1-1 rmp1-1* (*rmp1-1*) (transgenes in parentheses) strain was recovered and used as recipient in transformation experiments. As described above, the *Nsi*I-*Dra*III transforming fragment contains the *leu1*$^+$ gene, which replaces *rmp1-1*. Thus, the transformants were screened for leucine prototrophy. They were then tested by PCR analysis for the integration of the relevant fragment at the *rmp1* locus. For this purpose, we used primer 30, localized upstream of the *Sac*I restriction site in the genomic sequence (Figure 1), and a primer localized in the expected neighboring 3′ region of the *leu1* gene. This test was performed on pools containing mycelia from 10 transformants, followed by a sib-selection procedure applied on positive pools, using a method developed by E. Coppin (personal communication). Three transformants among the 193 tested gave an amplification of a fragment with the expected size. A second PCR test was then performed using primer 24 (Figure 1) and a primer localized in the expected neighboring 5′ region of the *leu1* gene. For two of these transformants, it was established that the *rmp1-1* sequence was not replaced by the deleted *rmp1* copy. The unique candidate, Δ*rmp1 leu1-1* (*rmp1-1*) (*leu1*$^+$), was crossed with a *leu1-1 rmp1-2* strain, and 12 asci were analyzed to control the segregation of the leu$^+$ phenotype. As expected, due to the tight genetic link between *rmp1* and the *mat* locus, the leu$^+$ ascospores were all *mat*−, but only those carrying the ectopic copy of *rmp1-1* were able to germinate. The inability of Δ*rmp1* ascospores to germinate was deduced from ascal analyses with respect to segregation of the *mat* locus (linked to *rmp1*) and of the selective marker associated with the *rmp1-1* transgenic copy. A Δ*rmp1* (*rmp1-1*) strain devoid of the *leu1-1* mutation was obtained by crosses.

To isolate a balanced heterocaryon bearing Δ*rmp1* in one of its two nuclei, a *rmp1-2 mat*+ *leu1-1* strain was crossed with a Δ*rmp1 mat*− *leu1*$^+$ (*rmp1-1*) strain (MB15, Table 2), in which the *rmp1-1* ectopic copy was carried by pPMB (Table 1). Marker segregation was controlled in asci issued from this cross. Candidate heterocaryotic strains of the genotype being sought (issued from binucleate ascospores) were submitted to genetic analysis to confirm their genotype, *i.e.*, Δ*rmp1 mat*− *leu1*$^+$/*rmp1-2 mat*+ *leu1-1*.

Sequencing: Genomic DNA was prepared according to Lecellier and Silar (1994). The localization of primers used for PCR performed on genomic DNAs is shown in Figure 1. In contrast to the other alleles, *rmp1-1* was sequenced from subclones of the 5.2-kb *Sac*I fragment, initially by the universal and reverse primers, followed by oligonucleotides deduced from the sequence. Cloned fragments (*rmp1-1*) or PCR products (other *rmp1* alleles) were sequenced using the ABI PRISM ready reaction dye deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), with automatic sequencing machines (373 A or 310 DNA sequencer; Applied Biosystems).

Cytological analyses: Processing of cells for meiocyte staining was as described previously (Berteaux-Lecellier *et al.* 1998). Strains expressing GFP were observed with a Zeiss Axioplan photomicroscope. Fluorescence images were captured by a CDD Princeton camera system. Mitochondria were stained with the vital mitochodrion-specific dye 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI; Sigma), according to the procedure described previously (Jamet-Vierny *et al.* 1997), after growth of the relevant strains at 27° and 37°.

Database search analyses: In addition to the general databases, we used specific databases for *N. crassa* (http://pedant.gsf.de/), *Aspergillus fumigatus* (http://www.sanger.ac.uk/Projects/A_fumigatus/), *Magnaporthe grisea* (http://www.genome.wi.mit.edu/annotation/fungi/magnaporthe/), *Histoplasma capsulatum* (http://genome.wustl.edu/projects/hcapsulatum/), *Schizosaccharomyces pombe* (http://www.sanger.ac.uk/Projects/S_pombe/), and *S. cerevisiae* (http://genome-www.stanford.edu/Saccharomyces/).

RESULTS

The *rmp1* gene was cloned by complementation of the *rmp1-2* defects: Two possible cloning strategies were available for *rmp1*. The first was based on the dominance of *rmp1-1* over *rmp1-2* (Contamine *et al.* 1996): *AS1-4* strains, heterocaryotic for the two *rmp1* alleles, show a very short life span, characteristic of *rmp1-1*. Thus, transformation of a *AS1-4 rmp1-2* strain with a cosmidic library issued from a *rmp1-1* strain should lead to the recovery of the *rmp1-1* sequence in transformants displaying a short life span. Unfortunately, *AS1-4 rmp1-2* strains sporadically exhibit short life spans (Contamine *et al.* 1996). Consequently, this transformation procedure implies long and tedious analyses to eliminate false-positive transformants, especially because we use the sib-selection method (Akins and Lambowiz 1985). The second strategy was less time consuming and based on the following observation. *AS1*$^+$ strains bearing the *rmp1-1* and *rmp1-2* alleles, respectively, exhibit a mycelial difference, which cannot be seen in a *AS1-4* context: after 2 days of growth at 37°, *AS1*$^+$ *rmp1-1* thalli show aerial hyphae while *AS1*$^+$ *rmp1-2* thalli do not. Since the *rmp1-1* haplotype is again dominant over the *rmp1-2* haplotype, the gene responsible for aerial hyphae formation at 37° could be cloned by complementation of a *rmp1-2* strain. Although we knew that this gene could be either *rmp1* or another tightly linked gene, we chose the second strategy. A genomic library, constructed from a *rmp1-1* strain (see materials and methods), was used to transform *AS1*$^+$ *rmp1-2* with pools containing 96 cosmids. Restoration of aerial hyphae at 37° was observed in 2 transformants (among 250) in the eighth cosmid pool tested. Crosses between these 2 transformants and a *AS1-4* strain suggested that the integrated cosmid carried the *rmp1-1* allele because all the *AS1-4 rmp1-2* ascospores carrying the cosmidic marker gave rise to thalli exhibiting a short life span. The mycelial phenotype was used to identify the relevant cosmid through successive rounds of sib selection. In the final step (in the single cosmid transformation), we obtained 10 transformants (among 20) exhibiting the expected phenotype, aerial hyphae at 37°. The gene responsible

TABLE 1

Plasmids used in this study

Plasmids	Comments	Selectable phenotype	Source
pCB1004	Used to create pHSS	Hygro^R	Carroll *et al.* (1994)
pPaBle	Used to create pPMB	Phleo^R	Coppin and Debuchy (2000)
pHSS	From pCB1004; contains the *Sac*I 5.2-kb fragment encompassing *rmp1-1*	Hygro^R	This study
pPMB	From pPaBle; contains the *Mlu*I-*Bsrg*I 3.4-kb fragment encompassing *rmp1-1*	Phleo^R	This study
pHfs	From pHSS with *rmp1-1* bearing a frameshift mutation	Hygro^R	This study
pLSS	From pHSS deleted of the *hph* gene and in which *rmp1-1* was replaced by the *leu1*$^+$ gene	Leu$^+$	This study
pHSS*	pHSS deleted of its polylinker	Hygro^R	This study
pHG	pHSS* in which *rmp1-1* bears the R165G missense mutation	Hygro^R	This study
pHN	pHSS* in which *rmp1-1* bears the UAG stop codon at position 982 (E982*)	Hygro^R	This study
pHNG	pHSS* carrying a reconstructed *rmp1-2* allele (*rmp1-1* bearing R165G and E982*)	Hygro^R	This study
pHRGFP	From pHSS* with *rmp1-1::GFP* gene fusion	Hygro^R	This study
pHNRGFP	From pHRGFP with *rmp1-1::GFP* bearing a deletion of 21 codons (Δ3-23) in the 5′ part of the ORF	Hygro^R	This study

See materials and methods for further comments. *, indicates, according to the international nomenclature, that the codon for glutamic acid (E) in position 982 is replaced by a stop codon (see Figures 1–3).

for this phenotype was localized in the cosmid according to the procedure developed by Turcq *et al.* (1990). The ability to complement the mycelial phenotype of a *rmp1-2* strain was found in a 5.2-kb *Sac*I fragment, which was cloned into pCB1004, giving rise to pHSS (Table 1). A smaller fragment, *Mlu*I-*Bsrg*I of 3.4 kb (Figure 1), bearing the same complementing ability, was cloned into pPaBle and named pPMB (Table 1). Two lines of evidence demonstrated that the gene involved in the mycelial phenotype seen in the *AS1*$^+$ context and the gene involved in the longevity feature in the *AS1-4* background are one and the same gene, namely *rmp1*. First, when transformants carrying either of the two plasmids (pHSS and pPMB) were crossed with *AS1-4*, *AS1-4 rmp1-2* strains recovered in the progeny and bearing the transgenic sequences exhibited the short life span characteristic of *AS1-4 rmp1-1*. Second, when a *AS1-4 rmp1-2* strain was directly transformed with pHSS, this short-life-span phenotype was observed in 20 (of 54) transformants. Crosses of 3 transformants confirmed that the relevant phenotype was linked to the transgenic sequence. These data permitted us to use the mycelial phenotype as an easy marker of *rmp1* in some of the further analyses.

The sequence of the complementing 5.2-kb *Sac*I fragment revealed an uninterrupted ORF encoding a puta-

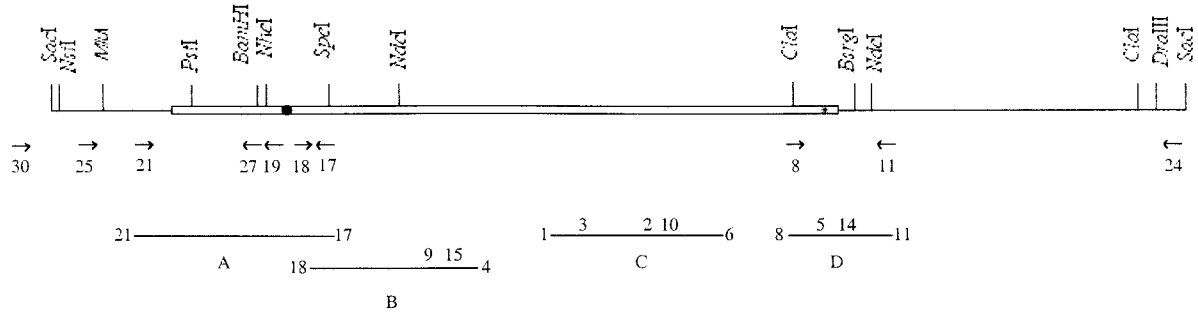

Figure 1.—Partial restriction map of the *rmp1* chromosomal region (5.2-kb *Sac*I fragment) and oligonucleotides used for PCR or sequencing. Only relevant restriction sites used in this study are shown. The open rectangle corresponds to the *rmp1* ORF. The black circle and the star indicate, respectively, the position of the missense (R165G) and nonsense (E982*) mutations present in *rmp1-2*, compared to *rmp1-1*. Arrows show the localization, name, and direction of oligonucleotides used for PCR experiments performed in plasmid constructions and for construct sequencing. Line fragments A, B, C, and D represent the PCR amplifications performed for sequencing all the natural alleles of *rmp1* (with the exception of *rmp1-1*). The numbers above the lines indicate the oligonucleotides used to obtain the junction between B and C and between C and D. The junction between B and C regions was achieved using the following pairs of oligonucleotides: 15-3, 9-10, and 9-3, depending on the alleles. The junction between C and D was obtained by the following pairs of oligonucleotides: 2-5, 2-11, 2-14, and 10-5, depending on the alleles.

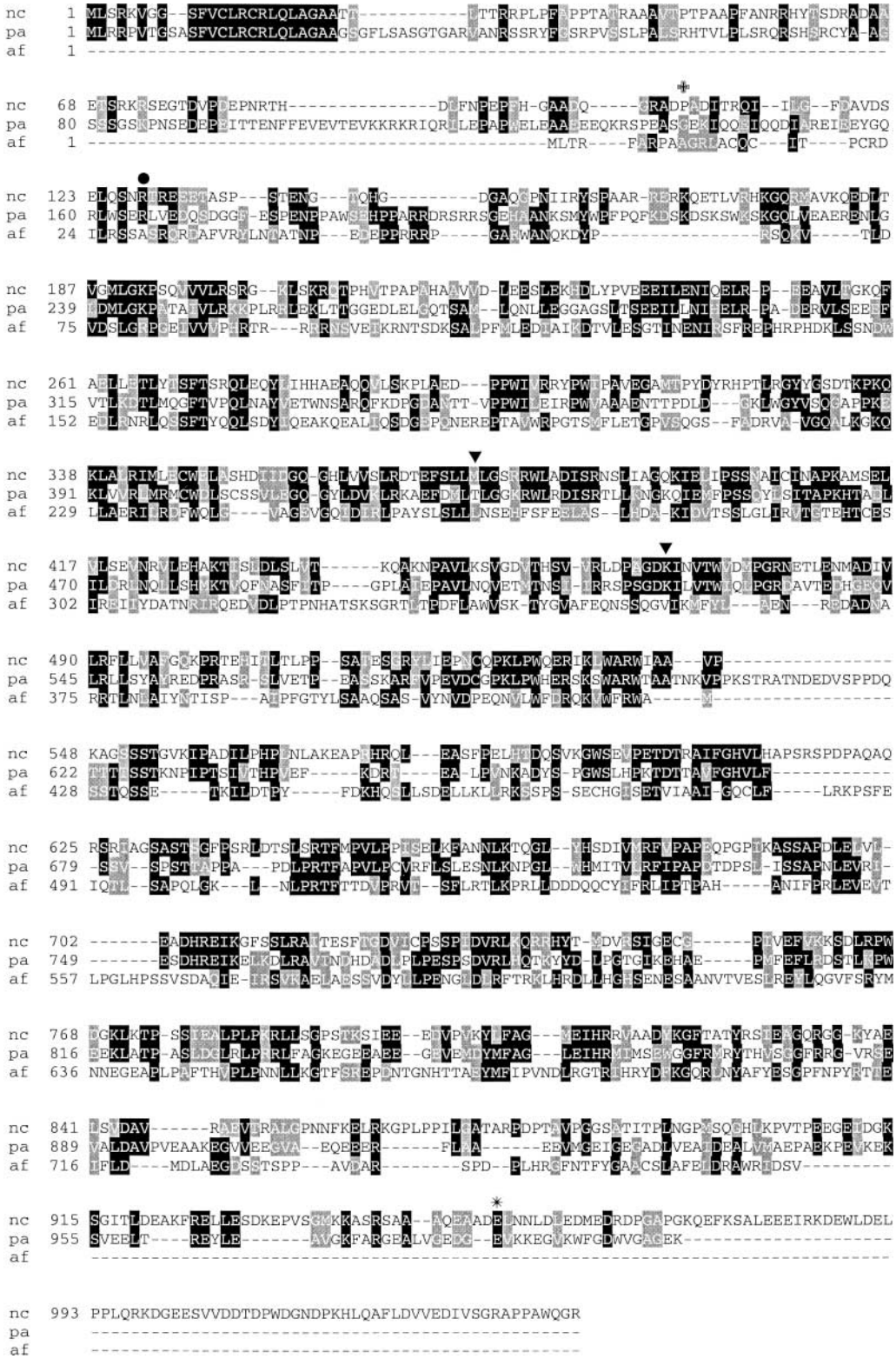

Figure 2.—Comparison of the predicted sequence of RMP1 (pa) with its putative homologs in *N. crassa* (nc) and *A. fumigatus* (af). Identical amino acids are in a black background; similar amino acids are in a shaded background. The positions of the introns in the *N. crassa* sequence are indicated by a triangle. The position of the frameshift mutation created in pHfs (Table 1) is indicated by a cross. Positions of the missense (R165G) and nonsense (UAG) mutations in the *rmp1-2* allele are indicated by a circle and a star, respectively. The alignment was obtained by the PIMA 1.4 algorithm (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The DDBJ/EMBL/GenBank accession number of the *rmp1-1* sequence is AJ581793. The *N. crassa* and *A. fumigatus* sequences can be found at the relevant web sites indicated in MATERIALS AND METHODS.

tive protein of 1000 amino acids. To demonstrate that this ORF corresponded to the *rmp1-1* allele, a frameshift mutation was introduced at codon position 138 (Figure 2), which simultaneously created a stop codon at the same position (see MATERIALS AND METHODS). The corresponding construct (pHfs, Table 1) was introduced into a *AS1*$^{+}$ *rmp1-2* strain and the transformants checked for their mycelial phenotype after growth at 37°: 24 of 28 transformants exhibited the flat mycelium characteristic of *AS1*$^{+}$ *rmp1-2* whereas 4 transformants exhibited aerial hyphae. Genetic analysis of these transformants suggested that this phenotype could be explained by the

reconstruction of a *rmp1-1* allele through recombination between the transgenic sequence and the endogenous *rmp1-2* allele. In the control experiment, performed with the unmodified form of *rmp1-1*, complementation of the recipient strain was observed in most transformants (*i.e.*, 28 of 33). Thus, the fact that a frameshift mutation at the beginning of the ORF led to loss of its complementing ability demonstrated that this ORF was *rmp1-1*.

***In silico* analysis of the RMP1 protein and its putative homologs:** Database searches using the BLAST program revealed that the RMP1 protein had putative homologs in four fungal species for which genomic sequences were available: *N. crassa*, *M. grisea*, *A. fumigatus*, and *H. capsulatum*. Identity percentages are 39, 32, 25, and 27% (BESTFIT program) between RMP1 and the four other sequences, respectively. Furthermore, the parameter termed "quality of the alignment" was compared with the average quality of 100 alignments of random permutations in each case. The reduced deviation (Z parameter), calculated as (cognate quality − average quality/standard deviation of quality of random permutations), was 205, 106, 21, and 22 for the four combinations, respectively. All these values, including the lowest, are highly significant (Slonimski and Brouillet 1993). Overall, these data reflect the phylogeny of those fungi. All five are filamentous ascomycetes. *P. anserina*, *N. crassa*, and *M. grisea* are Pyrenomycetes while *A. fumigatus* and *H. capsulatum* are Plectomycetes. In addition, *P. anserina* and *N. crassa* are closer to one another than to *M. grisea*. The PSORT program (Nakai 2000) disclosed nuclear localization signals (NLS) in three of the five sequences. RMP1 contains three monopartite and one bipartite signal while *N. crassa* and *A. fumigatus* sequences contain one and two monopartite signals, respectively. Further studies (see below) did not help us to understand why RMP1 contains NLS. Interestingly, the unique feature shared by the five sequences is a mitochondrial targeting peptide (mTP) as predicted by the Target P program (Emanuelsson *et al.* 2000). An alignment of RMP1 and its putative homologs in *N. crassa* and *A. fumigatus* is shown in Figure 2.

***rmp1* is an essential gene:** A *mat*− strain bearing the Δ*rmp1* allele was obtained as described in materials and methods. The recipient strain, used for the transformation-mediated gene replacement, carried an ectopic copy (SS2, Table 2) of the *rmp1-1* allele carried by pHSS (Table 1). As expected, the primary transformants and the purified strains, recovered through crosses, which exhibited the Δ*rmp1* (*rmp1-1*) genotype (transgene in parentheses), displayed a *rmp1-1* phenotype. These crosses also gave rise to ascospores bearing the Δ*rmp1* allele without the complementing ectopic *rmp1-1* copy, but none was viable. Careful microscopic examination revealed that Δ*rmp1* ascospores did in fact form one or two small filaments, which did not continue growth even after transfer to different media (M2, M1, MR) at any temperature tested (18°, 27°, 37°). This Δ*rmp1* defect was confirmed repeatedly in the numerous crosses performed in this study (see below): among >150 Δ*rmp1* ascospores tested, either *AS1*$^+$ or *AS1-4*, none was viable even on a germination medium enriched with yeast extract. This lethality can be complemented by an ectopic insertion, not only of pHSS but also of pPMB, which, respectively, carry the 5.2- and the 3.2-kb fragments encompassing the *rmp1-1* allele (Tables 1 and 2). In contrast, the pHfs plasmid, which carries *rmp1-1* with a frameshift mutation (see Table 1 and above), was unable to complement the lethality of Δ*rmp*. To test if Δ*rmp1* could be complemented by a functional *rmp1* allele present in another nucleus, a heterocaryotic strain was constructed (see materials and methods). In such a heterocaryotic Δ*rmp1 mat*− *leu1*$^+$/*rmp1-2 mat*+ *leu1-1* strain, the *rmp1-2* nucleus should complement the lethality of the Δ*rmp1* nucleus, which, in turn, complements the auxotrophy due to the *leu1-1* mutation present in the *rmp1-2* nucleus. Indeed, ascospores bearing this genotype germinate normally and the issuing strains grow on minimal medium. This result indicates clearly that Δ*rmp1* internuclear complementation takes place.

To ensure that Δ*rmp1* lethality was not restricted to the ascospore germination stage, we used the heterocaryotic strain to examine the regeneration capacity of Δ*rmp1* protoplasts. Three types of protoplasts are expected: those in which the heterocaryotic state is maintained, homocaryotic protoplasts carrying the *leu1-1* nucleus, and, finally, homocaryotic protoplasts containing the Δ*rmp1* nucleus. As shown in Table 3, on leucine-supplemented medium, 92% of the regenerating protoplasts were homocaryotic *leu1-1*; this indicates a clear loss of the heterocaryotic state. In contrast, on medium devoid of leucine, 89% of the regenerating protoplasts were heterocaryotic. The lack of homocaryotic Δ*rmp1* protoplasts in this experiment, in which the number of Δ*rmp1* nuclei was high enough to be easily detected, shows clearly that they did not regenerate. Interestingly, a few *leu1-1* protoplasts were recovered from the selective medium. Their regeneration ability can be explained by sufficient amounts of the LEU1 protein provided in the original heterocaryon. In contrast, none of the 98 protoplasts tested from both regenerating conditions displayed the *mat*− (Δ*rmp1*) genotype. It is not excluded that the two degenerative thalli of unknown genotype recovered in this experiment contained a Δ*rmp1* nucleus, but even if so, they were not able to regenerate beyond a small filament, like the Δ*rmp1* ascospores (see above). These observations provide strong evidence that both Δ*rmp1* protoplasts and Δ*rmp1* ascospores are unable to give rise to viable thalli and thus that *rmp1* is an essential gene.

Wild-type *rmp1* alleles are polymorphic: The *rmp1* gene is tightly linked to the *mat* locus: the genetic distance between *mat* and *rmp1* is ~0.25 cM (Contamine

TABLE 2

Expression of different forms of *rmp1* in the Δ*rmp1* background

	Transgene		Context and phenotype	
Plasmid[a]	Name	Genotype	*AS1*$^+$ (aerial hyphae)[b]	*AS1-4* longevity (cm)[c]
pHSS	SS2	*rmp1-1*	++	1–3[d]
pHMB	MB15	*rmp1-1*	++	1–3[d]
pHG	G3	*rmp1-R165G*	++	1–3[d]
pHNG	NG4	*rmp1-2*	−	>118[e]
	NG8	*rmp1-2*	+	>89[f]
pHN	N4	*rmp1-E982**	−	ND
	N21	*rmp1-E982**	±	>124[e]
pHRGFP	RGFP2	*rmp1-1::GFP*	++	1–3[d]
pHNRGFP	NRGFP2	*rmp1-1*(Δ*3-23*)*::GFP*	−	>129[g]

ND, not determined.

[a] See Table 1 for more details. Note that *rmp1-2* was reconstructed from *rmp1-1*.

[b] Formation of aerial hyphae is observed after growth at 37°. The three symbols ++, +, and ± describe the thickness of aerial hyphae in comparison to the *AS1*$^+$ *rmp1-1* reference strain (++); − indicates absence of aerial hyphae.

[c] See MATERIALS AND METHODS.

[d] Short longevities were determined on 5 (SS2), 18 (MB15), 4(G3), and 7 (RGFP2) subcultures issued from one ascospore of each genotype. These values do not differ from those obtained with the reference *AS1-4 rmp1-1* strain.

[e] Longevities of the NG4 and N21 strains were measured in the same experiment. In the first case, 10 subcultures, issued from three ascospores were used; only 1 subculture stopped growing (at 9 cm). In the second case, 18 subcultures, issued from four ascospores were used; only 1 subculture stopped growing (at 66 cm). In this experiment, the *AS1-4 rmp1-2* reference strain showed a mean life span of >124 cm (16 subcultures issued from four ascospores; 1 subculture stopped growing at 68 cm).

[f] Measurements were made on 15 subcultures, issued from four ascospores. Only 1 subculture was still growing (>171 cm) at the end of the study. In this experiment, the *AS1-4 rmp1-2* reference strain had a mean life span of >135 cm (6 subcultures, issued from two ascospores, with 2 subcultures still growing >171 cm).

[g] Measurements were made on 11 subcultures issued from four ascospores. Six subcultures were still growing when the experiment ended. In this series, the shortest longevity was observed at 88 cm. In this experiment, the *AS1-4 rmp1-2* reference strain displayed a mean life span of >150 cm (no dead subculture among 17 issued from five ascospores).

et al. 1996). The *rmp1-1* and *rmp1-2* alleles are linked to *mat*− and *mat*+, respectively. Genetic experiments have also previously shown that isolates of *P. anserina* and *P. comata* (closely related to *P. anserina*) can be separated into two classes. The first class (*e.g.*, s) bears two *rmp1* alleles similar to *rmp1-1* and *rmp1-2* with respect to life spans in a *AS1-4* context: the *rmp1-1*-like and *rmp1-2*-like alleles linked to *mat*− and *mat*+, respectively (and thus like our reference wild-type S strain). In the second class, the isolates (*e.g.*, A and *P. comata*) carry a single type of *rmp1* allele, which exhibits the features characteristic of *rmp1-1*: these alleles confer a short life span to a *AS1-4* strain regardless of the associated mating type (CONTAMINE *et al.* 1996). We thus decided to sequence the *rmp1* gene from *mat*+ and *mat*− strains of the s and A isolates and of *P. comata* (Pc), in addition to the *rmp1-2* allele of our S strain (see MATERIALS AND METHODS and Figure 1).

The data are reported in Figure 3. Each *rmp1* allele was named according to both its origin (S, s, A, or Pc) and its presence in the *mat*− (number 1) or *mat*+ (number 2) haplotypes. The *rmp1-1* allele (S1) was used as reference. Overall, four main conclusions can be drawn. First, a number of changes, scattered over the entire ORF, are found when the *P. comata rmp1* alleles are compared to those of the *P. anserina* isolates. Second, five sites appear polymorphic among the *P. anserina* isolates. Third, in all cases, roughly half of the substitutions are nonsynonymous. Finally and importantly, comparison between S2 (*i.e.*, *rmp1-2*) and s2, on one hand, and all other *rmp1* alleles on the other hand, discloses the molecular differences responsible for the functional differences between *rmp1-1* and *rmp1-2*. S2 and s2 share a premature stop (UAG) codon at position 982, which yields a protein lacking its last 19 amino acids. The two alleles also differ from S1 (*i.e.*, *rmp1-1*) and s1 by a missense mutation at position 165. However, this R165G is also found in A2 and in the two *rmp1* alleles of *P. comata*, which display the functional status of *rmp1-1*. Therefore, the longevity differences observed between *AS1-4 rmp1-1* and *AS1-4 rmp1-2* can be due to either the stop codon alone or its association with the R165G substitution.

Dissection of the *rmp1-2* allele: An ectopic copy of *rmp1-1* fully complements *rmp1-2* and Δ*rmp1*. When the reference criteria are tested, namely aerial hyphae at

TABLE 3

Protoplasts recovered from the heterocaryotic strain *Δrmp1 mat− leu1⁺/rmp1-2 mat+ leu1-1*

	Protoplasts recovered	
Regeneration medium[a]	Genotypes[b]	No.[c]
M1 + leu	*mat+/mat−*	3 (8)
	leu1-1 mat+	34 (92)
M1	*mat+/mat−*	54 (89)
	leu1-1 mat+	5 (8)
	Unknown[d]	2 (3)

Numbers in parentheses are percentages.

[a] M1 is the minimal medium used for protoplast regeneration. M1 + leu is supplemented with leucine (100 μg/ml).

[b] Only the relevant genetic markers are shown. Note that Δ*rmp1* is present in the *mat−* nucleus.

[c] The number of protoplasts capable of regeneration is much higher on M1 + leu than on M1. Few were tested from M1 + leu. See MATERIALS AND METHODS for more details.

[d] Degenerative thalli, which did not grow beyond the M1 plates.

37° in the *AS1⁺* context and a very short life span in the *AS1-4* context, *AS1⁺ rmp1-2* (*rmp1-1*) or *AS1⁺* Δ*rmp1* (*rmp1-1*) do not differ from *AS1⁺ rmp1-1*, and *AS1-4 rmp1-2* (*rmp1-1*) or *AS1-4* Δ*rmp1* (*rmp1-1*) do not differ from *AS1-4 rmp1-1* (see above and Table 2). Sequence comparisons of *rmp1-1* and *rmp1-2* revealed two nonsynonymous substitutions leading to a missense (R165G) and a nonsense (E982*) mutation in *rmp1-2*. To address the question of the roles of these mutations in the phenotypic features due to *rmp1-2*, three plasmids derived from pHSS were constructed. They contain a *rmp1* allele, which bears the missense mutation alone (pHG), the nonsense mutation alone (pHN), or both mutations (pHNG; Table 1 and MATERIALS AND METHODS). These plasmids were introduced singly into a *rmp1-2* recipient strain. Mycelia of the primary transformants were carefully examined for the presence or absence of aerial hyphae after growth at 37°. One transformant representative of each phenotypic class was then crossed to introduce the new *rmp1* alleles into the Δ*rmp1* context with or without the *AS1-4* mutation.

The data are reported in Table 2. The *rmp1-R165G* allele is not different from *rmp1-1*. (i) Of the 10 primary transformants recovered, 9 exhibited aerial hyphae after growth at 37°. (ii) The Δ*rmp1* (*rmp1-R165G*) strains exhibited the two characteristic features of *rmp1-1* (Table 2). The results obtained with the reconstructed *rmp1-2* allele (carried by pHNG) and *rmp1-E982** (carried by pHN) are less clear-cut. With both plasmids, the primary transformants showed two different phenotypes. About half of the transformants exhibited no aerial hyphae after growth at 37°, as observed for the *rmp1-2* allele. The second half showed aerial hyphae that were less dense than those of *rmp1-1* strains, a phenotype which is more or less halfway between those of *rmp1-1* and *rmp1-2*. This surprising phenotype was maintained through crosses. It was dominant over *rmp1-2*, as observed in the primary transformants, and recessive with respect to *rmp1-1*. It was also observed in a Δ*rmp1* context (Table 2). Although these data remain unexplained, the important point is that the *AS1-4* strains bearing either one of the two constructs (pHNG or pHN) exhibited the high longevity characteristic of *rmp1-2* (Table 2). On the basis of this criterion, we conclude that the *E982** mutation alone leads to the same phenotype as that of the original *rmp1-2* allele.

Cellular location of the RMP1-GFP protein shows that the *rmp1* gene is developmentally regulated: To localize RMP1, the protein encoded by *rmp1-1* was tagged exactly at its carboxy terminus with green fluorescent protein (GFP; see MATERIALS AND METHODS) and the relevant construct was introduced into a *rmp1-2* recipient strain. Primary transformants were screened, initially for the presence of aerial hyphae at 37°, and then for GFP expression. Aerial hyphae were observed in half of the transformants (8/18). However, their density varied among transformants, suggesting that complementation of *rmp1-2* was more or less efficient. The level of complementation correlated with the intensity of GFP fluorescence. One transformant, which exhibited the highest expression of *rmp1-1::GFP*, was chosen for further analysis. As shown in Table 2, the transgene (RGFP2) displays the features of a *bona fide rmp1-1* allele: it fully complements Δ*rmp1* and confers a very short life span to *AS1-4* strains.

GFP expression was examined during vegetative growth of the Δ*rmp1* (*rmp1-1::GFP*) strain RGFP2 and over the sexual cycle in perithecia obtained by crossing this strain (used as the female partner) with a *rmp1-2* strain. During early mycelial growth (<3 days), GFP labeling was not found along all filaments but only along the filaments giving rise to microconidia (conidiophores) in those surrounding the female organs (ascogonia) and in the ascogonia. In contrast, fluorescence was detected in microconidia (Figure 4, A and B) and in the vegetative filaments (Figure 4C) only after 3 days of growth. The GFP signal was first seen in the apical cells and then extended to the entire thallus (data not shown). The snake-like forms of the fluorescent bodies are mitochondria, as demonstrated by staining with the vital mitochondrion-specific dye DASPMI (compare Figures 4C and 5A). Note that DASPMI is currently used to specifically label mitochondria since the pioneer work of SOGO and YAFFE (1994) in yeast. We have previously shown that this dye works well in Podospora (JAMET-VIERNY *et al.* 1997). In contrast to the mycelium, which exhibited a GFP staining restricted to mitochondria, the ascogonia showed both cytosolic and punctate or reticular bright staining (see Figure 4D). Similarly, such a punctate pattern was also seen in all paraphysae (sterile filaments intermingled with asci) formed inside the perithecia (Figure 4E). To identify the nature of

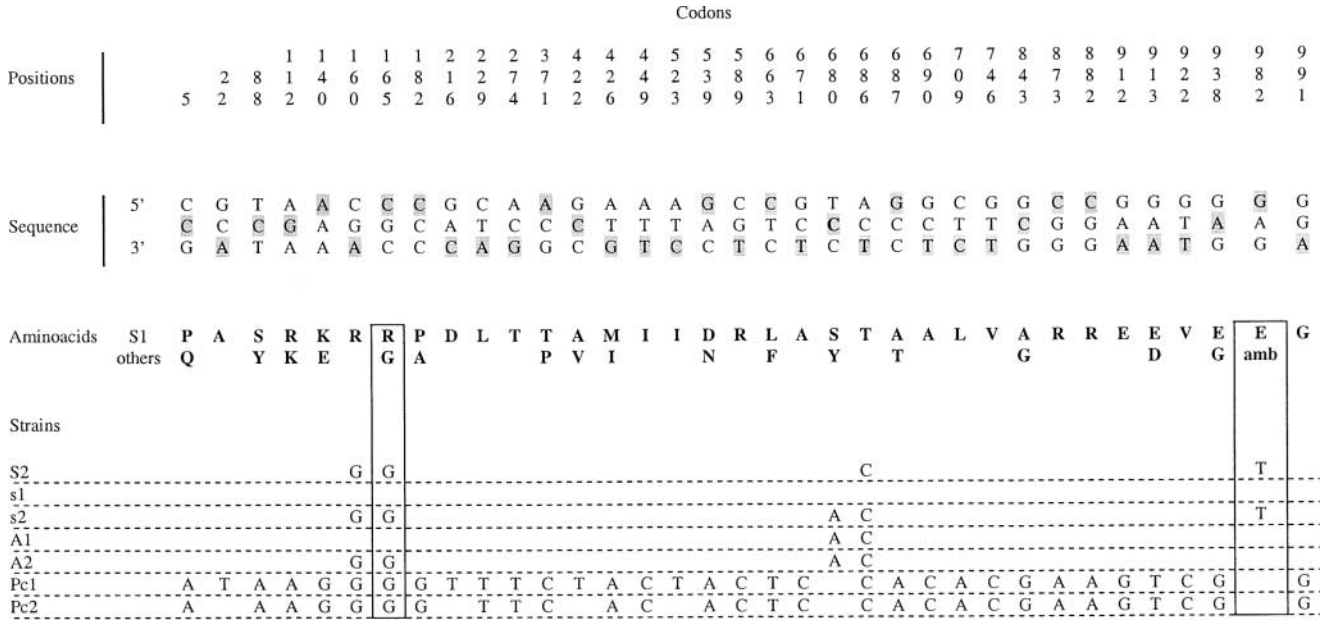

FIGURE 3.—Polymorphism of different wild-type *rmp1* alleles. Polymorphic codons are numbered vertically by position (from 5 to 991). The sequence of each codon is presented vertically, according to the *rmp1-1* sequence. The polymorphic base is indicated by a shaded background. The first line with boldface type gives the amino acids translated from the *rmp1-1* sequence. The boldface characters of the second line correspond to the residues translated from the sequences that differ from *rmp1-1*. Each strain from which *rmp1* was sequenced is named according to its origin (S, s, and A are wild-type isolates of *P. anserina*, with S being the reference strain used; Pc stands for *P. comata*) and its mating type (1, *mat*−; 2, *mat*+). For each *rmp1* allele, nucleotide differences with respect to *rmp1-1* are shown under the relevant codon positions. For instance, the CGA codon at position 160 in *rmp1-1* is changed to CGG in five *rmp1* alleles. This synonymous substitution maintains an arg residue at this position in the polypeptide. In contrast, the CGC codon at position 165 is changed into GGC in the same five alleles. This nonsynonymous substitution leads to a gly rather than an arg residue in the polypeptides (R165G). Codon GAG at position 982 is changed to a UAG (amber) codon in S2 and s2. Note that S1 and S2 give the sequence of *rmp1-1* and *rmp1-2*, respectively, and that s2 is a *rmp1-2*-like allele with respect to *AS1-4* longevity. The two positions in which both *rmp1-2* and s2 differ from *rmp1-1* are framed.

the punctate staining, these cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and DASPMI. The fluorescent bodies observed in the cytoplasm are mitochondria, as seen by DAPI (compare Figure 4, E and F) and by DASPMI (data not shown). In contrast, RMP1-GFP was never detected in the nuclei. During perithecial development, RMP1-GFP appears only in heterocaryotic basal cells, which contain numerous copies of the two parental nuclei. In contrast, no signal was detected in the dicaryotic cells (croziers), which emerge from the basal cells and contain one copy of each parental nucleus. Similarly, no fluorescence was seen in asci undergoing caryogamy, meiosis, and postmeiotic mitoses (data not shown). However, the signal reappeared in the ascospores. In early ascospores with young membranes, RMP1-GFP was seen in both mitochondria and cytosol (Figure 4, G and H). It is noteworthy that only the mitochondria of the ascospores are fluorescent and not those of the ascal cytoplasm in which the ascospores are formed. In more mature ascospores, only the cytosolic signal remained visible (Figure 4, I and J); it disappears in fully mature ascospores. We ruled out the possibility that the cytosolic signal comes from autofluorescence for the following reason. Structures that do not contain the *rmp1-1::GFP* construct do not show fluorescence. This is especially striking in the ascospores because the crosses are heterozygous with respect to this construct, which therefore segregates in the ascospores. The relevant cross was performed several times and we can estimate that at least 30 ascospores have been examined in each case (early, more mature, and fully mature ascospores).

These observations lead to three main conclusions. First, the *rmp1* gene is developmentally regulated. Second, the RMP1-GFP protein is seen in both cytosol and mitochondria. Third, depending on cell type and developmental stage, RMP1 either can be seen in both compartments or is restricted to one or the other compartment.

Analysis of strains expressing a RMP1 protein devoid of its putative mitochondrial targeting peptide: To further analyze the role of the RMP1 putative mitochondrial-targeting peptide in both localization and protein function, we constructed a *rmp1-1* allele lacking codons 3-23 and fused to the GFP sequence (MATERIALS AND METHODS). This construct was introduced in a *rmp1-2* recipient strain. All the transformants (30/30) exhibited the *rmp1-2* phenotype (no aerial hyphae at 37°).

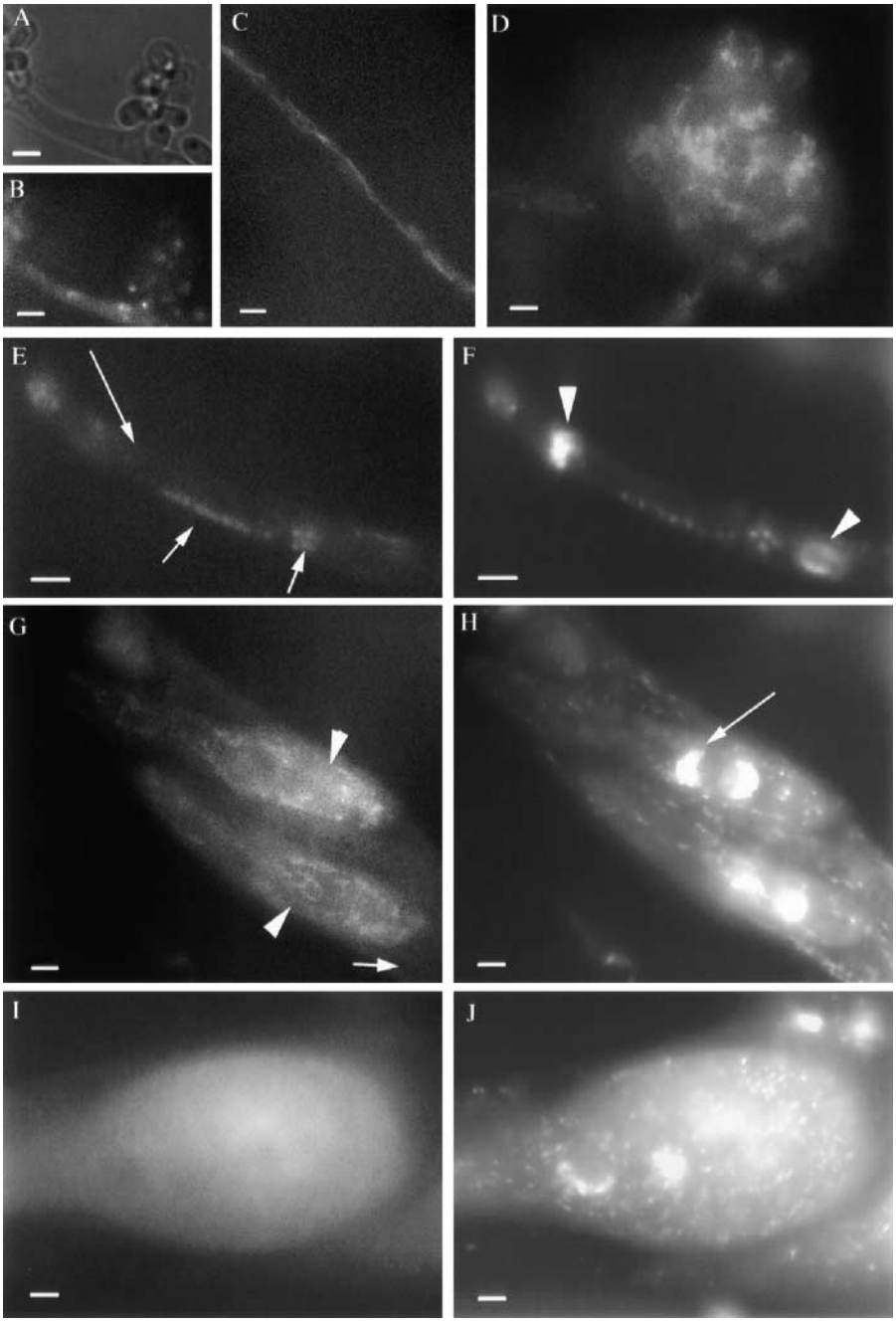

FIGURE 4.—RMP1-GFP localization in Δ*rmp1* (*rmp1-1::GFP*) during the vegetative and sexual cycles following fertilization with a *rmp1-2* partner. Note that photographs in A–D were performed on living cells. (A and B) RMP1-GFP staining in microconidia: (A) group of eight microconidia observed in bright light; (B) the same group observed through a GFP filter. Note that each microconidium shows one bright fluorescent spot. (C) In vegetative filaments after 3 days of growth, RMP1-GFP is visible in snake-like organelles, similar in size and shape to the mitochondria stained with DASPMI (Figure 5A). (D) RMP1-GFP is always visible in ascogonia (female organ). Note the reticular pattern superimposed on the cytosolic fluorescence. (E and F) RMP1-GFP fluorescence (E) and DAPI staining (F) in a paraphysa. The two large DAPI spots correspond to nuclei (arrowheads) and the small dots to mtDNA nucleoids. Note the complete overlap between the GFP signal and the DAPI staining in the mitochondria (short arrows) and the absence of GFP staining in the nuclei (arrow). GFP (G) and DAPI staining (H) of two very young ascospores. Note that the structures in G are also mitochondria because they can be superimposed on the DAPI staining in H. As in paraphysae, this overlap is not a strict colocalization, which means that RMP1 is not specifically associated with the nucleoids; only the mitochondria located in the two ascospores (arrowheads) are stained by RMP1-GFP, while the mitochondria present in the surrounding ascus are not (short arrow). Also, as seen for paraphysae, no RMP1-GFP signal is found in the nuclei (arrow points to one nucleus). (I and J) In a nearly mature ascospore, the GFP signal (I) is exclusively cytosolic. Note also that the surrounding cytoplasm is not stained, although several mitochondria are present, as revealed by the DAPI staining in J. Bar, 5 μm.

This suggests that the construct does not complement this *rmp1-2* defect. One of the transformants (NRGFP2, Table 2) was crossed to strains of interest to introduce the *rmp1-1* (Δ3-23)*::GFP* allele in all other possible genetic backgrounds.

Strikingly, this allele was able to complement the lethality of Δ*rmp1*, indicating that it was at least partially functional. GFP staining was followed in a Δ*rmp1* strain bearing the *rmp1-1* (Δ*3-23*)*::GFP* transgene. Contrary to RMP1-GFP, the staining was solely cytosolic or absent: mitochondria were never seen labeled. These results are very important. They demonstrate first that the RMP1 mTP is functional and second that, although the RMP1 protein is essential, its mTP is dispensable. To test if mitochondria lacking the RMP1 protein were different in morphology and/or distribution, mitochondria of a *rmp1-1* and a Δ*rmp1* [*rmp1-1*(Δ*3-23*)*::GFP*] strain were stained with DASPMI. As shown in Figure 5, mitochondria of the two strains were very similar in number, distribution, and mostly also in size. However, a few enlarged mitochondria were systematically seen in the mutant strain (roughly one per cell), whatever the growth temperature (27° and 37°). In contrast, such giant organelles were not observed in wild-type strains (compare Figure 5, A and B; see also JAMET-VIERNY *et al.* 1997).

Although viable, the Δ*rmp1* strains bearing the *rmp1-1*

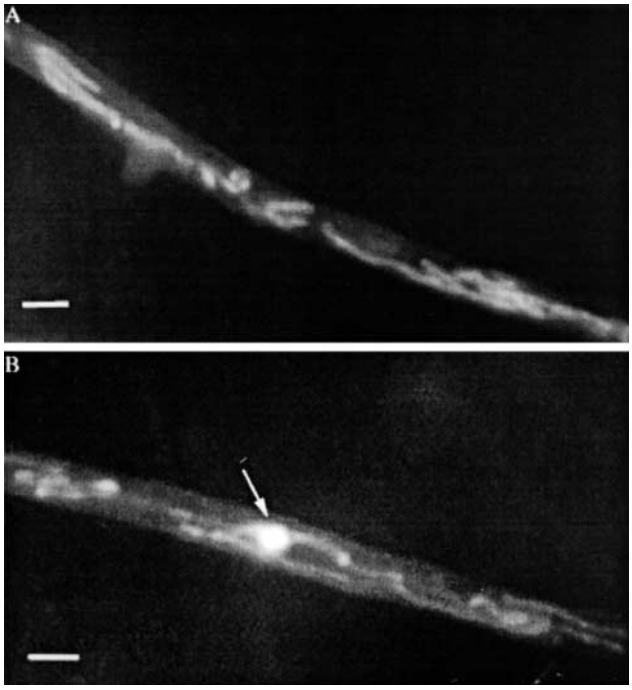

FIGURE 5.—Phenotypes of mitochondria in *rmp1-1* (A) and Δ*rmp1* [*rmp1-1*(Δ3-23)*::GFP*] (B) vegetative filaments. Mitochondria are stained with DASPMI. Arrow points to an enlarged mitochondria. Note that the giant mitochondria are much brighter than the normal mitochondria as previously observed in *PaTOM70* and *PaMDM10* mutants (JAMET-VIERNY *et al.* 1997). Bar, 5 μm.

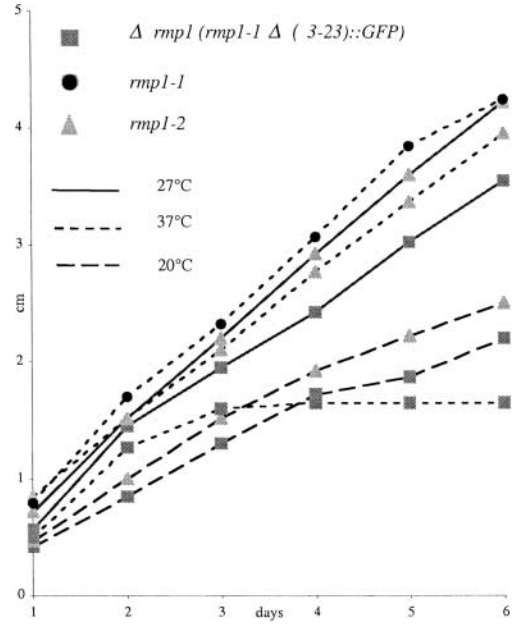

FIGURE 6.—Growth curves of Δ*rmp1* [*rmp1-1*(Δ3-23)*::GFP*], *rmp1-2*, and *rmp1-1* at three different temperatures. The growth curves of *rmp1-1* at 27° and 20° have been omitted because at these temperatures this strain exhibits the same growth rate as *rmp1-2*. Similarly, the growth curves of *rmp1-1* [*rmp1-1*(Δ3-23)*::GFP*] and *rmp1-2* [*rmp1-1*(Δ3-23)*::GFP*] are identical to those of *rmp1-1* and *rmp1-2*, respectively.

(Δ*3-23*)*::GFP* construct are not completely wild type. They display no aerial hyphae, not only at 37° (Table 2) but also at 27° (this phenotype is visible immediately following ascospore germination). Furthermore, as shown in Figure 6, their growth rates differ from those of the control strains at the three temperatures tested. At 37°, their growth was even arrested after a few days. However, the strains did not die: they resumed growth after transfer to 27°. In the course of these studies, it was observed that the life span of a *AS1*$^+$ Δ*rmp1* strain carrying the *rmp1-1* (Δ*3-23*)*::GFP* transgene was increased twofold in comparison with the reference strains. All these features are recessive: the *AS1*$^+$ *rmp1-1* and *rmp1-2* strains carrying the construct exhibit the phenotypic properties of the *rmp1-1* and *rmp1-2* reference strains, respectively, including their growth rates (see legend of Figure 6) and their life spans (data not shown).

Interestingly, when introduced in a *AS1-4* Δ*rmp1* context, *rmp1-1*(Δ*3-23*)*::GFP* leads to a very long life span, not different from those characteristic of the reference *AS1-4 rmp1-2* strains (Table 2). The new *rmp1* allele is recessive with respect to *rmp1-1*, as is *rmp1-2*: *AS1-4 rmp1-1* strains, which bear the *rmp1-1*(Δ*3-23*)*::GFP* construct, exhibit the very short life span typical of *AS1-4 rmp1-1* (data not shown). In conclusion, deletion of codons 3–23 in the *rmp1-1* coding sequence creates a new, viable *rmp1* allele, which produces a RMP1 protein unable to enter mitochondria (at least in detectable amounts). Although this new allele shares some properties with *rmp1-2*, it also has its own features and cannot be considered simply as similar to *rmp1-2*.

Role of RMP1 during the sexual cycle: With the knowledge that *rmp1* is developmentally regulated, we analyzed in further detail the possible role of RMP1 during the sexual cycle. Δ*rmp1* nuclei can be maintained only in balanced heterocaryotic strains. The Δ*rmp1 mat*− *leu1*$^+$/*rmp1-2 mat*+ *leu1-1* strain (see above) is able to self-fertilize. However, it is impossible to determine if this strain produces Δ*rmp1* female organs in addition to those containing *rmp1-2* nuclei. In contrast, the spermatization method (MATERIALS AND METHODS) demonstrated that the heterocaryotic strain forms functional Δ*rmp1 mat*− microconidia able to fertilize a *mat*+ tester strain. Due to its viability, it was possible to directly address these questions for a Δ*rmp1* strain bearing the *rmp1-1*(Δ*3-23*)*::GFP* construct. In this case, functional microconidia and female organs are formed. These data lead to two conclusions. First, the *rmp1* gene plays no role in the fertilization ability of the microconidia. Second, although we do not know if RMP1 *per se* is dispensable for female organ differentiation, our results demonstrate that RMP1 without mTP is sufficient for their development.

In a second step, we examined the contents of perithecia issued from crosses involving a Δ*rmp1* partner with or without a transgenic form of *rmp1-1*. The results

TABLE 4

Sexual defects observed in perithecia issued from crosses implicating different alleles of *rmp1*

Genotypes of the male partners[a]	No. of asci				
	Total	Before ascospore formation		After ascospore formation	
		In meiosis[f]	Abortive[f]	Normal	Abnormal[g]
rmp1-1	313[d]	0	9 (3)	304	0
Δ*rmp1*[b]	227[e]	117 (50)	13 (6)	87	10 (10)
	241[d]	52 (20)	8 (3)	170	11 (6)
Δ*rmp1* (*rmp1-1*)[c]	510[d]	0	6 (1)	447	57 (11)
Δ*rmp1* (*rmp1-1::GPP*)[c]	387[e]	144 (37)	6 (1.5)	226	11 (4.5)
	367[d]	0	26 (7)	304	37 (11)
Δ*rmp1* [*rmp1-1*(Δ3-23)*::GFP*][c]	381[e]	60 (16)	67 (18)	167	87 (34)

[a] In all crosses, the female partner was a wild-type *rmp1-2 mat+* strain. Delay in ascospore ejection from perithecia was observed in the second and the fifth crosses.

[b] The Δ*rmp1* male gametes are issued from the heterocaryotic strain Δ*rmp1 mat− leu1*$^+$*/rmp1-2 mat+ leu1-1*.

[c] The transgenes are SS2, RGFP2, and NRGFP2 for (*rmp1-1*), (*rmp1-1::GPP*), and [*rmp1-1*(Δ3-23)*::GFP*], respectively (see Table 2).

[d] The contents of perithecia were examined the fifth day after fertilization.

[e] The contents of perithecia were examined the fourth day after fertilization.

[f] The numbers in parentheses give the percentage with respect to the total of asci.

[g] The numbers in parentheses give the percentage of asci with abnormal ascospores among sporulated asci.

are reported in Table 4 and lead to the following remarks. Abortive asci are present in all crosses including a wild-type cross (*rmp1-1* × *rmp1-2*). They represent a small percentage of total asci except when the Δ*rmp1* nucleus contains the *rmp1-1* (Δ3-23)*::GFP* construct: here, nearly 20% of asci are abortive. Asci containing abnormal ascospores are found in all crosses except in the wild-type control. In all cases, the abnormalities in shape and number of ascospores correlate with an abnormal distribution of nuclei (data not shown). This defect is always associated with the presence of a Δ*rmp1* nucleus in the crosses, whatever the transgenic sequence. However, while in all mutant crosses the percentage of asci with abnormal ascospores represents ~10% of all spored asci, this value attains one-third of the asci when the Δ*rmp1* nucleus bears the *rmp1-1* (Δ3-23)*::GFP* construct. Overall, our observations lead to three conclusions. First, defects observed when Δ*rmp1* is heterozygous in a cross suggest that the *rmp1* gene dosage likely plays a role. For instance, the RMP1 protein could be rate limiting for proper ascospore formation. Second, although the 5.2-kb *Sac*I fragment (Figure 1) fully complements Δ*rmp1* during vegetative growth, it is unable to complement its sporulation defect. One simple explanation is that a sequence required for the full expression of *rmp1* during sexual reproduction is lacking in this fragment. Finally and noteworthy, our results show that *rmp1-1* (Δ3-23)*::GFP*, whose vegetative features are recessive (see above), acts as a dominant negative allele during sexual reproduction. In other words, the extent of defects is higher in a cross heterozygous for this allele than in crosses heterozygous for Δ*rmp1*. Thus, it seems that a RMP1 protein devoid of its mTP is poisonous for the asci.

DISCUSSION

The *P. anserina* RMP1 protein exhibits several noteworthy features with three intertwined facets. First, the function(s) of RMP1 is unknown and its putative homologs are, to date, found only in filamentous ascomycetes. Second, RMP1 is developmentally regulated: the RMP1-GFP fusion protein can be undetectable, cytosolic, and/or mitochondrial, depending on the cell type and the developmental stage. Third, RMP1 is essential but its mTP is dispensable.

***rmp1* encodes a protein whose putative homologs can be found only in filamentous ascomycetes:** RMP1 is a large protein that lacks recognizable motifs. This hampers understanding of its function. Furthermore, *rmp1* putative homologs have been found only in the genomes of filamentous (multicellular) ascomycetes. Three hypotheses can account for this situation. First, the *rmp1* function could be restricted to these fungi. The Woronin body is an example of a structure specific to filamentous ascomycetes; this specialized vesicle occludes septal pores when the filaments are damaged, thus avoiding cell death by preventing loss of cytoplasm. However, this function is not essential for growth: a lack of Woronin bodies is not lethal (Jedd and Chua 2000; Tenney *et al.* 2000). A systematic search for essential genes has been undertaken in *A. fumigatus* (Firon *et al.* 2002; Firon and d'Enfert 2002), but present reports have not yet revealed if some are specific to filamentous ascomycetes. In fact, to our knowledge, essential genes characterized in this evolutionary lineage are either involved in general, basic functions common to all organisms (*e.g.*, translation) or shared by the entire fungal kingdom; *i.e.*, homologs are also found in unicellular

ascomycetes (yeasts) and in basidiomycetes. For instance, inactivation of the gene encoding the catalytic subunit of glucan synthase is lethal not only in *A. fumigatus* (Firon *et al.* 2002) but also in *S. cerevisiae* (Mazur *et al.* 1995) and in *Cryptococcus neoformans* (Thompson *et al.* 1999). This gene is indeed specific to fungi for which β-(1-3) glucan is an essential component of the cell wall. With this viewpoint, *rmp1* could be the first essential gene specific to multicellular ascomycetes.

Second, the *rmp1* function could be widely distributed but, in other evolutionary lineages, ensured by a nonhomologous gene encoding a structurally different protein; this implies that *rmp1* and the other putative gene have no common ancestor. This situation is illustrated by the *thyA/thyX* genes, which both encode a protein with thymidylate synthase activity. However, the two proteins lack any sequence similarity and are not structurally related. With a few exceptions, *thyA* and *thyX* have mutually exclusive phylogenetic patterns (Myllykallio *et al.* 2002).

A third model also makes sense: the *rmp1* function could be widely distributed, but the corresponding gene evolves so rapidly that recognition of its homologs in distant species would be impaired. Such a hypothesis is supported by the weak similarities observed between RMP1 and its putative homologs in filamentous ascomycetes: they fall between 39 and 25% identity, and this is dependent on the phylogeny. In contrast, when 163 *P. anserina* putative coding sequences (located in the two regions surrounding the centromere of chromosome V) are compared with their putative homologs in *N. crassa*, the percentages of identity are centered on 60–70%, with nearly 90% of the proteins exhibiting >40% identity (Silar *et al.* 2003). In addition, seven nuclear genes encoding proteins with well-known mitochondrial functions have been characterized in *P. anserina*. The percentages of identity with their *N. crassa* homologs range from 47 to 80% (data not shown) with the exception of mtHMG1 (30%). In the latter case, the recognition of its *N. crassa* and mammalian putative homologs relies mainly on the presence of HMG-type DNA-binding domains (Dequard-Chablat and Alland 2002; M. Dequard-Chablat, personal communication). Thus, as for *mthmg1*, *rmp1* may belong to this class of genes, which encode proteins either weakly constrained in sequence evolution or subjected to positive selection. The high ratio of nonsynonymous *vs.* synonymous substitutions observed in *rmp1* does not permit us to distinguish between these two hypotheses. If there were a selection parameter, it might be driven by coevolution with a partner of the protein, *e.g.*, mtDNA for mtHMG1. It is noteworthy that, in contrast to mtHMG1 (Dequard-Chablat and Alland 2002), RMP1 does not colocalize with the mitochondrial nucleoids. This suggests that the relationship of *rmp1* to mtDNA integrity/transmission is indirect, as evidenced for several nuclear-mitochondrial genes in *S. cerevisiae* (reviewed in Contamine and Picard 2000).

Although all the pieces of the puzzle are still not in place to explain the evolutionary position of *rmp1*, an unsettling observation favors the third model. When the putative *H. capsulatum* homolog of RMP1 was used to question a general nonredundant database, a significant alignment (BLAST E value of 3×10^{-11}) was found with a hypothetical protein of *S. pombe*, SPAP8A3.14C. In contrast, no putative homologs were found when the same database was searched for RMP1 and its other fungal counterparts, using an E cutoff value of 1×10^{-4}. The *S. pombe* protein has a weak similarity to *S. cerevisiae* Sls1p, with an E value of 3×10^{-7}. Sls1p is a mitochondrial membrane protein required for respiration (Rouillard *et al.* 1996). It was recently proposed that this protein may play a key role in modulating the translation efficiency of mitochondrial mRNAs (Bryan *et al.* 2002). To date, we do not favor the idea that RMP1 and Sls1p might be homologous. Furthermore, the similarity between the *S. cerevisiae* and *S. pombe* proteins appears questionable (Z value: 9). However, one cannot exclude that the *H. capsulatum* homolog of RMP1 and the SPAP8A.14C sequence of *S. pombe* might bridge the gap between unicellular and multicellular ascomycetes in the case of a rapidly evolving gene. To elucidate this point, an understanding of the function(s) of both RMP1 and SPAP8A3.14C is required.

Expression of *rmp1* and subcellular localization of RMP1 are developmentally regulated: In addition to sequence analyses and comparisons, another approach to the function of *rmp1* was the study of its expression throughout the life cycle and the localization of its product. Our work clearly shows that *rmp1* expression is subject to spatial and temporal controls. This is true during both the vegetative and sexual cycles. In the vegetative mycelium and in the microconidia, the RMP1-GFP fusion is undetectable before 3 days of growth, while female organs formed during the same period are labeled. During sexual development, RMP1-GFP is found in certain cell types. To our knowledge, the results we present here show, for the first time, that nuclear genes encoding mitochondrial proteins can be developmentally regulated in filamentous fungi. With respect to *P. anserina*, we have previously demonstrated that staining of mitochondria with antibodies against the mitochondrial citrate synthase showed the same type of structures (regarding both their numbers and shape) as those observed with RMP1-GFP but the relevant *cit1* gene was not developmentally regulated (Ruprich-Robert *et al.* 2002). In contrast, such regulation was previously described in yeast and higher eukaryotes. In Drosophila, for instance, expression of the *fzo* gene, encoding a protein required for mitochondrial fusion during spermatogenesis, is restricted to the male germ line, while the *dmfn* gene, which encodes a protein of the same family, exhibits a broad expression pattern (Hwa *et al.*

2002 and references therein). This is a good illustration of how the expression pattern of a gene may indicate a specific or a general function. However, only a genetic analysis determined the precise role of *fzo* (Hales and Fuller 1997). Similarly, in *S. cerevisiae*, some nuclear genes with known roles in mitochondrial function are up- or downregulated during sporulation (*e.g.*, Chu *et al.* 1998; see also the *Saccharomyces* Genome Database) but the functional reasons for their expression pattern remain mostly unknown. With respect to *rmp1*, the regulation of expression seen at the protein level is especially complex. The reason that RMP1 is undetectable when the strain resumes growth, as well as in croziers, asci, and mature ascospores, is puzzling. Although RMP1 might be dispensable in croziers and asci, our data clearly demonstrate that it is essential for ascospore germination and protoplast regeneration. Two assumptions can explain this paradox. First, the protein *per se* may be needed at these stages. This implies that very low amounts of RMP1 (undetectable by GFP fluorescence) are sufficient to ensure its essential function. Second, RMP1 may seem dispensable at certain steps because it might have an enzymatic activity whose product accumulated during the preceding stages, *i.e.*, in the female organs before and after fertilization, in the stationary phase, and during ascospore maturation. This hypothetical product would ensure RMP1 function at subsequent stages. To explain the absence (or very low levels) of RMP1 at these critical stages, one can hypothesize a feedback control: high amounts of the RMP1 product would cause repression of *rmp1* until dilution of this product would permit derepression.

In addition to its complex regulation pattern, another remarkable feature of RMP1 is its localization in the cytosolic compartment, in mitochondria, or in both, depending on the cell type. Proteins encoded by a single gene and exhibiting both mitochondrial and cytosolic locations have been described in other organisms. However, in most cases, the two protein forms correspond to two different translation products in which the mTP is present or absent, due to alternative sites for initiation of transcription, alternative splicing, or alternative sites for initiation of translation. Yeast fumarase (Knox *et al.* 1998; Sass *et al.* 2001) and major adenylate kinase (Strobel *et al.* 2002) belong to a second class of proteins, whose dual location is ensured by a single translation product. RMP1 probably belongs to this class. This assumption is supported by the fact that a *rmp1-1* allele bearing a frameshift mutation between the first two ATGs of the ORF is unable to complement Δ*rmp1* lethality, whereas *rmp1-1*(Δ*3-23*), which encodes a mTP-truncated protein, complements the null allele. If there were two translation products, initiated at these two ATGs, the frameshift mutant should exhibit the properties of *rmp1-1*(Δ*3-23*). In yeast, two mechanisms have been proposed to explain the dual (cytosolic/mitochondrial) location of a single translation product. Both involve changes in protein conformation leading to an import-incompetent state. In the case of fumarase, cotranslational import of the precursor could follow two routes, one leading to mitochondrial localization after import completion and the other to a release of the protein back into the cytosol (Knox *et al.* 1998). With respect to the major adenylate kinase, its dual location is explained by a competition between folding (cytosolic location) and import (Strobel *et al.* 2002). One striking point with RMP1, which makes it a unique case, is that its subcellular location varies according to cell type and development. If the viewpoints proposed in *S. cerevisiae* are applied to RMP1, one could assume that cellular components or factors might differentially influence the ratio of import-competent *vs.* import-incompetent forms of RMP1, for instance, by post-translational modifications. In any case, although the extraordinary developmental and cellular patterns of RMP1 do not shed light on its function, they do provide an exciting model for further studies.

RMP1 is an essential protein, in which mTP is dispensable: A third way to shed light on *rmp1* function was careful examination of the phenotypic properties of the four alleles available. In addition to the two natural alleles, *rmp1-1* and *rmp1-2*, two new alleles were constructed: Δ*rmp1*, which is a complete deletion of the gene, and *rmp1-1*(Δ*3-23*), which carries a deletion of codons 3–23 and thus encodes a RMP1 protein without its mTP. In comparison with *rmp1-2*, *rmp1-1* is probably the fully functional allele. This conclusion is based mainly on the fact that *rmp1-2* shares phenotypic features with *rmp1-1*(Δ*3-23*). In a *AS1-4* context, both lead to a very long life span. In a *AS1*$^+$ background, the defects of *rmp1-2* are modest compared to those of *rmp1-1*(Δ*3-23*). In the first case, the strains lack aerial hyphae and display a slightly reduced growth rate at 37°. In the second case, these defects are also seen at 27° and the strains are heat sensitive. In addition, *AS1*$^+$ strains bearing the *rmp1-1*(Δ*3-23*) allele exhibit life spans twice those of the reference strains, and they show a few giant mitochondria at 27° and 37°. Finally, Δ*rmp1* is lethal. Therefore, one can conclude that *rmp1* is an essential gene but that absence (or a very low amount) of RMP1 in the mitochondria is compatible with viability, at least below 37°. Interestingly, some properties of *rmp1-1*(Δ*3-23*) are reminiscent of those previously observed in *P. anserina* when the mitochondrial metabolism is altered. For instance, a mutation in *PaTOM70*, encoding a protein implicated in the import of proteins from the cytosol into the mitochondria, leads to reduced formation of aerial hyphae, heat sensitivity, and striking increases in life spans of *AS1-4* and *AS1*$^+$ strains. In addition, strains bearing this mutation exhibit a few giant mitochondria (Jamet-Vierny *et al.* 1997; Contamine and Picard 1998).

The simplest hypothesis, with respect to the data reported above, is that RMP1 ensures the same essential function in both the cytosol and the mitochondria. If

this function implicates the synthesis of an unidentified compound (as assumed above), its production in the cytosol would supply limited amounts to the mitochondria, sufficient for viability but less than that seen when this compound is also produced within the organelle. It is noteworthy that growth of *rmp1-1*(*Δ3-23*) stops at 37° after 3 days. This is precisely at the time that RMP1 is found in mitochondria. Thus, as previously noted for *PaTOM70* (see above), the mitochondrial metabolism is probably rate limiting at high temperature. Furthermore, the differential localization of RMP1 observed throughout the life cycle would then reflect where and when the protein is necessary for optimal cellular function.

rmp1 is the fifth nuclear-mitochondrial gene identified in *P. anserina* by effects on the life spans of *AS1-4* strains; all five participate in multigenic control of the accumulation of the specific mtDNA-deleted molecules observed on this background. In contrast to the four genes previously characterized (see Introduction) whose functions are either suspected or well documented in other organisms, *rmp1* is an orphan gene. Its remarkable pattern of expression and its essential nature will generate further investigations. For example, the search for suppressors of either Δ*rmp1* lethality or *rmp1-1* (*Δ3-23*) heat sensitivity should help to disclose the function of *rmp1*. This might result in the identification of functional homologs of this puzzling gene in other evolutionary lineages.

We are much indebted to Robert Debuchy for his constant interest in this work and for valuable advice. We gratefully acknowledge Annie Sainsard-Chanet, Philippe Silar, and the people of our laboratory for helpful discussions and communication of unpublished data. We are also grateful to A. Billault, R. Debuchy, and P. Silar for providing a BAC containing the *rmp1-2* allele. Last but not least, we acknowledge the release of the fungal genome sequences to the international community. This work was supported by grants from Association Française contre les Myopathies.

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Communicating editor: M. Zolan