Two Nuclear Life Cycle–Regulated Genes Encode Interchangeable Subunits $c$ of Mitochondrial ATP Synthase in Podospora anserina

Michelle Déquard-Chablat,†1,2 Carole H. Sellem,†3 Pawel Golik,4,5 Frédérique Bidard,1,2 Alexandre Martos,6 Maïlis Bietenhader,6 Jean-Paul di Rago,*6 Annie Sainsard-Chanet,1,3 Sylvie Hermann-Le Denmat,*1,2,7 and Véronique Contamine*1,2

1Univ Paris-Sud, Orsay, France
2Institut de Génétique et Microbiologie (IGM), Centre National de la Recherche Scientifique, Orsay, France
3Centre National de la Recherche Scientifique, Centre de Génétique Moléculaire (CGM), Gif-sur-Yvette, France
4Faculty of Biology, Institute of Genetics and Biotechnology, University of Warsaw, Warsaw, Poland
5Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
6Institut de Biochimie et Génétique Cellulaires (IBGC), Centre National de la Recherche Scientifique, Université Victor Segalen Bordeaux2, Bordeaux, France
7École Normale Supérieure, Paris, France
†These authors contributed equally to this work.
*Corresponding author: E-mail: sylvie.hermann-ledenmat@igmors.u-psud.fr; veronique.contamine@igmors.u-psud.fr; JP.Dirago@ibgc.cnrs.fr.

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Abstract

An F$_1$F$_O$ ATP synthase in the inner mitochondrial membrane catalyzes the late steps of ATP production via the process of oxidative phosphorylation. A small protein subunit (subunit $c$ or ATP9) of this enzyme shows a substantial genetic diversity, and its gene can be found in both the mitochondrion and/or nucleus. In a representative set of 26 species of fungi for which the genomes have been entirely sequenced, we found five Atp9 gene repartitions. The phylogenetic distribution of nuclear and mitochondrial Atp9 genes suggests that their evolution has included two independent transfers to the nucleus followed by several independent episodes of the loss of the mitochondrial and/or nuclear gene. Interestingly, we found that in Podospora anserina, subunit $c$ is exclusively produced from two nuclear genes (PaAtp9-5 and PaAtp9-7), which display different expression profiles through the life cycle of the fungus. The PaAtp9-5 gene is specifically and strongly expressed in germinating ascospores, whereas PaAtp9-7 is mostly transcribed during sexual reproduction. Consistent with these observations, deletion of PaAtp9-5 is lethal, whereas PaAtp9-7 deletion strongly impairs ascospore production. The P. anserina PaAtp9-5 and PaAtp9-7 genes are therefore nonredundant. By swapping the 5' and 3' flanking regions between genes we demonstrated, however, that the PaAtp9 coding sequences are functionally interchangeable. These findings show that after transfer to the nucleus, the subunit $c$ gene in Podospora became a key target for the modulation of cellular energy metabolism according to the requirements of the life cycle.

Key words: ATP synthase, mitochondrion, subunit $c$, transcriptional regulation, gene partition, Podospora anserina.

Introduction

An F$_1$F$_O$ type ATP synthase catalyzes the final step of oxidative phosphorylation in mitochondria. This ATP synthase is composed of a hydrophobic F$_O$ subcomplex located in the inner mitochondrial membrane and involved in proton translocation, and a hydrophilic F$_1$ subcomplex that catalyzes ATP synthesis in the matrix space (Devenish et al. 2008; for review). The F$_1$ contains five subunits in a 3$a$/3$b$/1$c$/1$d$/1$e$ stoichiometry. The F$_O$ consists of several copies of subunit $c$ (ten copies in yeast Saccharomyces cerevisiae; Stock et al. 1999) arranged in a ring, and one copy of each of subunits $a$, $b$, $d$, $F_{6}$, and OSCP (Rak et al. 2009; for review). Subunits $b$, $d$, $F_{6}$, and OSCP form the “peripheral stalk” that lies to one side of the complex connecting the F$_O$ and the F$_1$ subcomplexes. The ATP synthase utilizes energy from the proton gradient established across the inner membrane during the transfer of electrons to oxygen by the respiratory complexes. The flow of protons through F$_O$ induces the rotation of the subunit $c$ ring and the $\gamma/\delta/\epsilon$ subcomplex (or “central stalk”) of F$_1$. This rotation leads to conformational changes to the catalytic sites in F$_1$, so as to favor the synthesis of ATP and its release into the mitochondrial matrix. Finally, additional subunits associated with F$_O$ (e, f, g, and A6L in human) contribute to complex assembly and/or oligomerization (Devenish et al. 2008).

Some of the genes encoding the mitochondrial ATP synthase are nuclear and some are mitochondrial (mt), except in a few organisms such as the green algae Chlamydomonas.
reinhardtii (Funes et al. 2002) and some parasite species (reviewed in Feagin 2000), where all the genes are nuclear. Mitochondrial genomes encode only a limited number of ATP synthase subunits, usually subunit α or 6 (Atp6 gene), subunit 6L (Atp8 gene), and subunit c or 9 (Atp9 gene). In some plants, subunits α (Atp1 gene) and b (Atp4 gene) are also encoded by the mt genome. In many early-branching animals, Atp9 is absent from mt genomes, although it is still present in mt genomes in demosponges except for Amphimedon queenslandica, for which there is evidence of a recent transposon-mediated transfer to the nuclear genome (Erpenbeck et al. 2007). The absence of the Atp8 gene from the mt genome of the placozoan Trichoplax adhaerens and that of the copepod Paracyclopsina nana was also recently reported (Signorovitch et al. 2007; Ki et al. 2009). In ciliates, only the Atp9 gene has been identified in the mt genome (de Graaf et al. 2009) and in trypanosomatids, for example, Trypanosoma brucei, only the Atp6 gene is mitochondrial (Feagin 2000). In fungi, subunit c is usually mitochondrial encoded, but it can also be encoded by both the nuclear and the mt genomes as in Ascomycota Neurospora crassa (van den Boogaart et al. 1982), Aspergillus nidulans (Brown et al. 1985), and Fusarium oxysporum (reviewed in Lavín et al. 2008). In animals, the Atp9 gene is nuclear and is absent from the mt genome (Anderson et al. 1981; De Grassi et al. 2006).

In the human genome, Atp9 is represented by a family of three nuclear genes encoding isoforms of subunit c (P1, P2, and P3). All three share an identical mature sequence of 76 amino acids, but have different mitochondrial targeting sequences (MTSs) (Dyer and Walker 1993; De Grassi et al. 2006). The P3 isofrom has been found in all vertebrates analyzed, whereas P2 is common to all mammals and P1 common to all mammals and Aves (De Grassi et al. 2006). Only one of these three isoforms, P1, is known to be actively regulated in mammals, and responds to cold acclimation (Houštěk et al. 1995; Andersson et al. 1997; Kramarova et al. 2008). Physiological regulation of Atp9 gene expression has been also reported in the fungus N. crassa. The nuclear gene is constitutively expressed during vegetative growth, and transcripts of the mt gene are only detectable, and accumulate, during conidia germination (van den Boogaart et al. 1982; Bittner-Eddy et al. 1994).

Here, we report the highly regulated expression of the two Atp9 genes identified in the nuclear genome of the filamentous fungus Podospora anserina (Ridder et al. 1991; Espagne et al. 2008; this study). The expression patterns of the two Atp9 genes differ substantially during the fungus life cycle. The temporal expression patterns correspond to the developmental defects associated with deletion of the genes. The polypeptides encoded display only 44% identity, but we show that they are functionally interchangeable subunits c of the P. anserina mitochondrial ATP synthase. We also reveal that the complex diversity of Atp9 gene distribution between mt and nuclear genomes in fungi is indicative of active gene transfer from the mitochondria to the nucleus for a gene encoding a highly hydrophobic polypeptide essential for ATP synthase activity.

**Materials and Methods**

**DNA and Protein Sequences**

The amino acid sequences of fungal Atp9 polypeptides were retrieved from various databases (see supplementary table S1, Supplementary Material online) using the following procedures. We first performed BlastP searches using the sequences of the P. anserina and S. cerevisiae proteins. If this failed to detect a homologous protein, we then performed TBLastN searches at the NCBI Web site (whole-genome shotgun reads database wgs), the PEDANT3 Web site and the Broad Institute (individual genomes at http://www.broad.mit.edu/science/data#). Database annotation was manually modified for four retrieved sequences to correct the length and position of intronic regions (supplementary materials and methods and supplementary table S1, Supplementary Material online).

**Phylogenetic Analysis**

Atp9 protein sequences were aligned using MUSCLE 3.7 (Edgar 2004). To estimate phylogenetic relationships, the protein sequences were analyzed using the maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods. For ML analysis, the PhyML program run from the SeaView interface was used (Guindon and Gascuel 2003; Gouy et al. 2010). The number of variable sites and across site rate variation was optimized. Reliability of nodes was assessed by 1,000 bootstrap replicates. For MP analysis, PAUP* (Swofford 2003) version 4.0b10 was used, with 1,000 bootstrap replicates to assess the reliability of nodes. BI analysis was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Trees were visualized using Dendroscope (Huson et al. 2007).

**P. anserina Strains, Growth Conditions, and Transformation**

Strains used in this study were all derived from the S wild-type strain (Rizet 1952) to ensure a homogenous genetic background. For homologous recombination, we used the phleomycin-resistant strain ΔPaKu70 (El-Khoury et al. 2008). Details concerning standard culture conditions, media, protoplast formation, protoplast transformation, and genetic methods are available at http://podospora.igmors.u-psud.fr. The standard growth medium and ascospore germination medium used were M2 and G, respectively. Agar plates were used to collect projected ascospores. When necessary, hygromycin (Euromedex, Soufflweyersheim, France), nourseothricin (Werner Bio-Agents, Jena, Germany) or phleomycin (Euromedex, Soufflweyersheim, France) were added to the protoplast regeneration medium at a concentration of 100, 50, and 10 µg/ml, respectively.
Deletions of the PaAtp9-5 (Δ5) and PaAtp9-7 (Δ7) Genes

Deletions of the PaAtp9-5 and PaAtp9-7 genes were performed by gene replacement with a nourseothricin-resistance and a hygromycin-resistance cassette, respectively. As inactivation of the PaAtp9-5 gene may be lethal, prior deletion of the PaAtp9-5 gene, we constructed a wild-type strain containing an ectopic copy of the gene (hereafter the S5 transgene). Polymerase chain reaction (PCR) amplification, cloning steps, and strain construction are described in supplementary materials and methods, and all primers used are listed in supplementary table S2 (Supplementary Material online).

Construction of Chimeric S5 and S7 Transgenes

Chimeric S5 and S7 fragments were obtained by fusion-PCR amplification to produce a PaAtp9-7 coding sequence (CDS) flanked by S5 (585 bp) and S3’ (739 bp) regions of PaAtp9-5 (S5 transgene) and a PaAtp9-5 CDS flanked by S5’ (1,456 bp) and S3’ (1,403 bp) regions of PaAtp9-7 (S7 transgene). PCR amplification steps and strain construction are described in supplementary materials and methods (Supplementary Material online).

Analyses of PaAtp9-5 and PaAtp9-7 Gene Expression by Real-Time Quantitative PCR

The expression level of the two PaAtp9 genes was determined along the life cycle of the wild-type strain grown at 27 °C (fig. 2A). At least three biological replicates were done for each point of gene expression analysis. Samples were collected as described below and stored at −80 °C until total RNA extraction. Ejected mature ascospores (G0) were recovered on agar plates for 5 days. For the G8 and G16 samples (the germination stage), ascospores were first retrieved on agar plates covered with celophane layers, which were then transferred to G medium to promote initiation of germination; samples were collected after 8 and 16 h. For the G24 and G48 samples, ascospores were recovered directly from G medium covered with celophane layers and mycelium was collected from germinating ascospores after growth for 24 and 48 h, respectively. For growth stage kinetics, mycelium from 48-h culture on M2 was minced in liquid M2 medium and 150 μl of the suspension was laid on Petri dishes containing M2 medium. After growth for 4 days, bands (0.6 mm) of mycelium were scraped from the apical growing zone (A; see fig. 2A) and from more mature zones (B, C, and D areas corresponding to approximately 2-, 3-, and 4-day-old mycelium, respectively; fig. 2A). Preparation of samples during sexual reproduction will be fully detailed elsewhere (Bidard F, Imbeaud S, Ichanté JL, Mucchielli MH, Debuchy R, Berteaux-Lecellier V, in preparation). Briefly, a female of mating type mat− and a male mat+ were crossed on M2 Petri dish covered with sterile cheesecloth (nylon cloth). Plates were exposed to light to promote perithecia development. Developing perithecia were then collected by scraping at the indicated times after fertilization (fig. 2A). R0 corresponds to mycelium after 96 h of growth in M2 under light and just before fertilization.

All samples (40–80 mg of ascospores or mycelium) were frozen in liquid nitrogen and ground for 1 min at 2,600 revolutions per minute using a Mikro-Dismembrator (Sartorius Stedim France, Aubagne, France). Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s protocol that includes a DNase I treatment. RNA integrity was checked by measuring optical density and agarose gel electrophoresis. Complementary DNA was synthesized from 2 μg of total RNA using the SuperScript III First Strand kit (Invitrogen, Cergy Pontoise, France) and oligo (dT)20 primer. Real-time PCR measurements were determined using a LightCycler 2.0 system (Roche Diagnostics GmbH, Mannheim, Germany) and the LightCycler FastStart DNA Master Plus SYBR Green I kit. Experimental conditions of real-time amplification reaction including normalization are described in supplementary materials and methods (Supplementary Material online).

Results

Distribution of Atp9 Gene in Nuclear and Mitochondrial Genomes of Fungi

The mt genome of the filamentous fungus P. anserina lacks an Atp9 CDS (Cummings et al. 1990), but a nuclear gene coding for the subunit c of mitochondrial ATP synthase was identified several years ago (Ridder et al. 1991). The genome sequence of P. anserina was recently reported (Espagne et al. 2008) and surprisingly contains a second nuclear gene encoding a polypeptide homologous to the subunit c of ATP synthase. The two coding sequences (formally Pa_5_9140 and Pa_7_20 and hereafter referred to as PaAtp9-5 and PaAtp9-7) are on chromosomes 5 and 7 and both contain two introns. PaAtp9-5 codes for a polypeptide of 144 amino acids and PaAtp9-7, for a polypeptide of 147 amino acids, with 44% overall sequence identity between the two. The identity is 66% in the putative mature part of the protein.

Identification of two Atp9 genes in the nuclear genome of P. anserina prompted us to look for Atp9 genes in different species representative of fungi. We selected 26 species for which both complete nuclear and complete mt genome sequences were available (table 1; supplementary table S1, Supplementary Material online). We also included the Dothideomycetes Pyrenophora tritici-repentis and the Sordariomycetes Chaetomium globosum and Magnaporthe grisea for which supercontigs of mt genomes were considered. Most of the 26 species are members of the phyla Ascomycota and Basidiomycota that include most (98%) described fungal species (James et al. 2006; Hibbett et al. 2007). In Basidiomycota, Zygomycota, and Chytridiomycota, there is a single Atp9 gene, in all cases in the mt genome. The Atp9 gene location and copy number in Ascomycota are highly diverse (table 1). In Saccharomycotina and Taphrinomycotina, there is only a single Atp9 gene in the mt genome. In Pezizomycotina, containing the vast majority...
of filamentous fruit body–producing species including *P. anserina*, the *Atp9* gene is differently distributed between the nuclear and mt genome in different species, and the copy number is between 1 and 3 (table 1 and fig. 1D) with five different *Atp9* gene distributions: 1) a single gene in the mt genome (Onygenales); 2) a single gene in the nuclear genome (Pleosporales); 3) one copy in each of the mt and nuclear genomes (Eurotiales, Hypocreales, and Sordariales, *N. crassa*, and *Sordaria macrospora*); 4) two copies in the nuclear genome (Sordariales, *P. anserina*, *Cha. globosum*, and *M. grisea*); and 5) two copies in the nuclear genome and one in the mt genome (Capnodiales, *Mycosphaerella graminicola*). This diversity is indicative of an active process of *Atp9* gene transfer from the mitochondria to the nucleus during the evolution of filamentous fungi, probably near the root of the Pezizomycotina subphylum, followed by gene loss in some lineages (see fig. 1D and Discussion).

Alignments of the putative mature part of the 36 proteins and alignments of the putative MTSs of the 15 proteins encoded in the nucleus are shown separately in figure 1A and B. The mature part of each nuclearly encoded protein was considered to be the sequence that aligned with the complete sequence of mitochondrially encoded polypeptides. The alignments are available in Supplementary Material online.

### Table 1. Distribution of the *Atp9* Gene in Nuclear and Mitochondrial Genomes in Fungi.

<table>
<thead>
<tr>
<th>Species Class</th>
<th>Species Name Abbreviation</th>
<th>Nuclear Genome</th>
<th>Mitochondrial Genome</th>
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<tbody>
<tr>
<td><strong>Ascomycota</strong></td>
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<tr>
<td>Pezizomycotina</td>
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<tr>
<td>Eurotiomycetes</td>
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<td></td>
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<tr>
<td>Eurotiales</td>
<td><em>Aspergillus nidulans</em></td>
<td>ASPNI</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>Penicillium marneffei</em></td>
<td>PENMA</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Onygenales</td>
<td><em>Paracoccidioides brasiliensis</em></td>
<td>PARBR</td>
<td>–</td>
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<tr>
<td></td>
<td><em>Trichophyton rubrum</em></td>
<td>TRIRU</td>
<td>–</td>
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<tr>
<td>Sordariomycetes</td>
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<tr>
<td>Hypocreales</td>
<td><em>Fusarium graminearum</em></td>
<td>FUSGR</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sordariales</td>
<td><em>Neurospora crassa</em></td>
<td>NEUCR</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td><em>Sordaria macrospora</em></td>
<td>SORMA</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td><em>Podospora anserina</em></td>
<td>PODAN</td>
<td>2</td>
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<tr>
<td></td>
<td><em>Chaetomium globosum</em></td>
<td>CHAGL</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td><em>Magnaportha grisea</em></td>
<td>MAGGR</td>
<td>2</td>
</tr>
<tr>
<td>Dothideomycetes</td>
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<tr>
<td>Capnodiales</td>
<td><em>Mycosphaerella graminicola</em></td>
<td>MYCGR</td>
<td>2</td>
</tr>
<tr>
<td>Pleosporales</td>
<td><em>Pyrenophora tritici-repentis</em></td>
<td>PYRTR</td>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>Stagonospora nodorum</em></td>
<td>STANO</td>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Saccharomycotina</td>
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<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>SACCE</td>
<td>–</td>
<td>+</td>
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<tr>
<td></td>
<td><em>Candida glabrata</em></td>
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<td>–</td>
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<td></td>
<td><em>Ashbya gossypii</em></td>
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<td>–</td>
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<td></td>
<td><em>Kluyveromyces lactis</em></td>
<td>KLULA</td>
<td>–</td>
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<td></td>
<td><em>Candida albicans</em></td>
<td>CANAL</td>
<td>–</td>
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<td></td>
<td><em>Yarrowia h ypolytica</em></td>
<td>YARLI</td>
<td>–</td>
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<tr>
<td>Taphrinomycotina</td>
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<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>SCHPO</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Basidiomycota</td>
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<tr>
<td>Agaricomycotina</td>
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<tr>
<td><em>Pleurotus ostreatus</em></td>
<td>PLEOS</td>
<td>–</td>
<td>+</td>
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<tr>
<td><em>Schizophyllum commune</em></td>
<td>SCHCO</td>
<td>–</td>
<td>+</td>
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<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>CRYNE</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Ustilagomycotina</td>
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<tr>
<td><em>Ustilago maydis</em></td>
<td>USTMA</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Zygomycota</td>
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<tr>
<td><em>Rhizopus oryzae</em></td>
<td>RHIOR</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Chytridiomycota</td>
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<tr>
<td><em>Allomyces macrogynus</em></td>
<td>ALLMA</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

**Note.**—<sup>a</sup> presence; <sup>b</sup> absence of an *Atp9* gene in the corresponding genome. Numbers indicate the copy number of the *Atp9* gene in the nuclear genome.

<sup>a</sup> Nuclear genes encoding PaATP9-5-like polypeptides (see fig. 1 and text for details).

<sup>b</sup> In this case, sequences of amplified cDNA fragments were checked (supplementary materials and methods).

<sup>c</sup> TBlastN analyses of supercontigs of the mitochondrial genome failed to reveal any *Atp9mt* open reading frame.

<sup>d</sup> Nuclear genes encoding PaATP9-7-like polypeptides (see fig. 1 and text for details).
Phylogenetic trees were inferred from mature protein sequences using BI, ML, and MP as described in Materials and Methods and supplementary materials and methods (Supplementary Material online). All the methods yielded an essentially identical topology, although support values were variable and generally low. It should, however, be remembered that the Atp9 sequences are relatively short and conservative, and even when much longer and more informative sequences offering a comprehensive sampling of fungal taxonomy are used, many of the deeper nodes of the fungal tree of life do not receive significant statistical support (James et al. 2006). The fact that different phylogenetic methods lead to the same conclusions gives the present study additional credibility. A representative tree obtained using BI is shown in figure 1C, and the ML and MP trees are shown in supplementary figure S1A and S1B (Supplementary Material online), respectively. Inclusion of the MTS sequence in the analysis also resulted in a very similar topology (BI tree in supplementary fig. S1C, Supplementary Material online).

The alignment and the phylogenetic tree reveal that all sequences deduced from the nuclear Atp9 genes fall into two distinct subgroups, and these two groups correspond to *P. anserina* Atp9-5 and Atp9-7 homologs (fig. 1C). These subgroups branch off deep in the tree and are close to another group consisting of the mitochondrial Atp9 sequences from Pezizomycotina, whereas the mitochondrial Atp9 sequences of Saccharomycotina, Taphrinomycotina, Basidiomycota, and basal fungi (Zygomycota and Chytridiomycota) form a separate clade. Such tree topology, together with obvious differences in MTS sequences, strongly suggest that the nuclear Atp9-5 and Atp9-7-like genes were independently acquired by separate events of transfer from the mitochondrial to the nuclear genome. The position of relevant groups in the tree and the distribution of these genes in distantly related members of Pezizomycotina strongly suggest that these two gene transfer events occurred very early in the evolution of filamentous fungi, before the divergence of the lineages leading to Eurotiomycetes, Sordariomycetes, and Dothideomycetes.

Phylogenetic relationships between the Atp9 sequences within each of the major groups (nuclear Atp9-5 like, nuclear Atp9-7 like, Pezizomycotina mitochondrial Atp9, non-Pezizomycotina mitochondrial Atp9; fig. 1C) are generally consistent with the established Assembling the Fungal Tree Of Life (AFTOL) classification of fungi (McLaughlin et al. 2009) and the phylogeny based on six gene sequences (James et al. 2006).

Divergences between the Atp9-5-like and Atp9-7-like mature sequences were mainly at the C and N terminal ends but there were also differences concerning residues in the transmembrane domains (TMHs; fig. 1A). The putative MTS of the Atp9-5 and Atp9-7 homologs are very different (fig. 1B). Also, the MTS of Atp9-5 homologs (including the functionally characterized MTS of *N. crassa*; Schmidt et al. 1984) are well conserved in sequence and length, whereas those of Atp9-7 homologs are more divergent. The differences between the MTS of the two groups are consistent with two independent events of gene transfer from the mitochondria to the nucleus rather than nuclear gene duplication (fig. 1D and Discussion).

For species with two copies of the Atp9 gene in the nuclear genome, one was Atp9-5 type and one Atp9-7 type (fig. 1A–C). For species with a single copy in the nuclear genome, it was either the Atp9-5 type (*N. crassa*, *Sa. macrospora*, *F. graminearum*, *A. nidulans*, and *Penicillium marneffei*) or the Atp9-7 type (*Pyr. tritici-repentis* and *Stagonospora nodorum*); all the species with only the Atp9-5 type gene contain a mitochondrial Atp9 gene, whereas those with the Atp9-7 type gene do not (table 1 and fig. 1D).

The presence of Atp9-5 and Atp9-7 homologs in both Sordariomycetes and Dothideomycetes, which are placed on distant parts of the Pezizomycotina tree (James et al. 2006; McLaughlin et al. 2009) strongly suggests that they were already present in the early ancestral filamentous fungi. The observed distribution of these sequences in modern genomes can be explained by independent gene loss events in different lineages (fig. 1D).

Nuclear-encoded subunit c proteins are also found outside fungi, for example, in *C. reinhardtii* (Funes et al. 2002) and in human (Dyer and Walker 1993; De Grassi et al. 2006). In order to determine their relationship to the Atp9-5 and Atp9-7-like polypeptides of Pezizomycotina, we included them in the phylogenetic analyses (supplementary fig. S1D, Supplementary Material online for the BI tree). The sequences from *C. reinhardtii* (CHLREIN-Atp9A and CHLREIN-Atp9B) and Homo sapiens (ATPSG1, ATPS6G2, and ATPS5G3, for isogenes P1, P2, and P3, respectively) were classified in the branch containing the mtDNA-encoded polypeptides from nonfilamentous fungi, which strongly suggests that the events that led to the transfer of the respective genes to the nuclear genomes were separate from those that occurred in Pezizomycotina.

PaAtp9-5 Deletion is Lethal, Whereas PaAtp9-7 Deletion Specifically Affects Ascospore Production

To study the functions of the PaAtp9-5 and PaAtp9-7 genes, we generated single deletions of each gene (hereafter ΔS and ΔT) as follows and analyzed the phenotypic effects on *P. anserina* life cycle (fig. 2A). Expressed sequence tag (EST) analysis found that in the wild-type strain, PaAtp9-5 were more abundant than PaAtp9-7 EST during ascospore germination, which is critical to trigger *P. anserina* life cycle (Espagne et al. 2008; Debuchy R, personal communication). Therefore, inactivation of the PaAtp9-5 gene may be deleterious. As a consequence, before constructing a PaAtp9-5 deletion, we introduced into the genome of the wild-type strain a Δs transgene that encompasses the PaAtp9-5 CDS with 5′ (585 bp) and 3′ (739 bp) flanking regions. The PaAtp9-5 CDS in the recipient PaAtp9-5 Δs transgenic strain was then replaced with a nourseothricin-resistance cassette to obtain a ΔS Δs strain. This was then crossed with the wild-type strain to segregate the Δs transgene, and the progeny was analyzed. Ascospores of the ΔS that did not express the transgene were smaller than wild-type and ΔS Δs ascospores;
Fig. 1. Alignment of ATP9 amino acid sequences encoded in fungi and their phylogenetic relationships. Abbreviations of species names are as indicated in table 1. Podospora anserina protein names are in bold. Conserved amino acids are boxed in black (identical) and gray (similar). (A) For proteins encoded in the nucleus, only the C terminal half of the sequence that aligns with mitochondrially encoded ATP9 proteins (ATP9mt) was taken into account. Numbering refers to the entire protein sequences. For each species with two nuclear Atp9 genes, the
they were also less pigmented and unable to initiate the germination stage, which triggers \textit{P. anserina} life cycle. Thus, deletion of \textit{PaAtp9-5} confers a lethal phenotype that cannot be complemented by \textit{PaAtp9-7}.

The \textit{AT7} strain was obtained directly by the replacement of the \textit{PaAtp9-7} CDS in the wild-type strain with a hygromycin-resistance cassette. This manipulation revealed that unlike \textit{PaAtp9-5}, \textit{PaAtp9-7} is not an essential gene. The sexual reproduction of the \textit{AT7} strain was then characterized. We compared the \textit{AT7} homozygous cross and the two reciprocal heterozygous crosses (i.e., female \textit{AT7} fertilized by wild-type male gametes and wild-type female fertilized by \textit{AT7} male gametes). The heterozygous cross of a wild-type female with a \textit{AT7} male resulted in no evident defect. The reciprocal heterozygous cross and the homozygous \textit{AT7} cross both gave similar defects: the perithecia differentiated from \textit{AT7} female organs were heterogeneous in size, unlike wild-type perithecia (fig. 2B). Microscopic observations revealed disorganization of the content of \textit{AT7} perithecia associated with a substantial deficit in ascospore formation. Indeed, very few, or even no, mature ascospores were produced (fig. 2B). The rare wild-type or \textit{AT7} mature ascospores formed in the \textit{AT7} perithecia (developed from heterozygous cross) were, however, normally pigmented and able to initiate ascospore germination (data not shown). Thus, the \textit{AT7} ascospores issued from the three crosses displayed no defects during germination or subsequent growth (growth stage).

In conclusion, the absence of the \textit{PaAtp9-7} gene from male gametes does not affect sexual reproduction, although its absence from female gametes is deleterious. \textit{PaAtp9-7} deletion thus has a maternal effect. Also, the \textit{PaAtp9-7} gene is necessary for efficient formation and production of ascospores but is inessential for germination. The \textit{P. anserina} \textit{PaAtp9-5} and \textit{PaAtp9-7} genes are clearly nonredundant: \textit{PaAtp9-5} is essential to trigger ascospore germination and \textit{PaAtp9-7} is important for sexual reproduction.

Expression of \textit{PaAtp9-5} and \textit{PaAtp9-7} Genes along \textit{P. anserina} Life Cycle

We analyzed the expression of the \textit{PaAtp9-5} and \textit{PaAtp9-7} genes by real-time quantitative PCR (RT-qPCR) experiments with total RNA extracted at different stages of the fungus life cycle (fig. 2A). RT-qPCR results (fig. 3) are reported as values for each \textit{PaAtp9} gene normalized to that for the reference gene \textit{PaPDF2}, which encodes protein phosphatase PP2A regulatory subunit A and is stably expressed (supplementary materials and methods, Supplementary Material online, for details).

In nongerminated ascospores (G0), \textit{PaAtp9-5} mRNA was thousand times more abundant than \textit{PaAtp9-7} mRNA (fig. 3A). During the germination process, which occurs on specific G medium, the \textit{PaAtp9-5} gene was strongly expressed, whereas \textit{PaAtp9-7} transcripts were barely detectable. A peak of \textit{PaAtp9-5} expression was observed during the initiation of ascospore germination (times 8 and 16 h; G8 and G16). At these times, \textit{PaAtp9-7} expression was at its lowest level. Thereafter (times 24 and 48 h; G24 and G48), the amount of \textit{PaAtp9-5} mRNA decreased, whereas that of \textit{PaAtp9-7} mRNA increased such that, after 48 h of germination \textit{PaAtp9-5} transcripts were only 20-fold more abundant than \textit{PaAtp9-7} transcripts (legend for fig. 3). The pattern of \textit{PaAtp9-5} expression was consistent with the failure of \textit{AT7} ascospores to initiate germination. Reciprocally, the very low abundance of \textit{PaAtp9-7} transcripts during germination is fully consistent with \textit{AT7} ascospores not displaying any germination defect.

Mycelium arising from 48-h germinating ascospore (G48) was used to inoculate M2 medium and grown for 4 days. Expression of the two genes was studied in four different regions of the mycelium: the actively growing zone (fig. 3B, zone A) and three quiescent areas corresponding to about 2-, 3-, and 4-day-old mycelium (zones B, C, and D, respectively; see also fig. 2A). Overall, the abundance of \textit{PaAtp9} transcripts was higher in the actively growing zone than other samples, and \textit{PaAtp9-7} transcripts are more abundant than \textit{PaAtp9-5} transcripts. The \textit{AT7} deletion strain did not display any abnormalities of mycelium growth during the corresponding stage. It appears that \textit{PaAtp9-7} transcripts, despite their low abundance, are sufficient to ensure mycelium growth.

We studied \textit{PaAtp9-5} and \textit{PaAtp9-7} gene expression in perithecia collected at various times after fertilization (see Materials and Methods). The samples analyzed correspond to: the time of fertilization (R0), the first developmental steps of fertilized female organs (R12), the formation and maturation of asci (R24 and R48), and the formation and maturation of ascospores (R72 and R96). At fertilization (R0), \textit{PaAtp9-7} mRNA was almost 40-fold more abundant than \textit{PaAtp9-5} mRNA (fig. 3C). During sexual reproduction, \textit{PaAtp9-7} mRNA remained more abundant, except when

nomenclature is as follows: ATP9A for \textit{PaATP9-5}-like polypeptide, ATP9B for \textit{PaATP9-7}-like polypeptide. If only one nuclear gene is present in the fungus, the nomenclature is ATP9. Protein sequences were aligned using MUSCLE 3.7 and manually adjusted. Dotted arrows indicate the position of the TMH. (B) Alignments of the N-terminal parts (putative MTS) of ATP9 proteins encoded by nuclear genes. Putative MTS of ATP9-5 and ATP9-7 homologs were aligned separately. The small vertical arrow indicates the proteolytic cleavage site determined experimentally for the \textit{N. crassa} ATP9 nuclearly encoded protein (NEUCR-ATP9). (C) Phylogenetic tree constructed for the alignment in (A) using the BI method (see Materials and Methods). Numbers next to selected nodes correspond to Bayesian posterior probabilities. (D) Evolutionary model for the origin of the \textit{ATp9} gene family in the Pezizomycotina subphylum. The topology of the tree, which is drawn according to the six-genre phylogenetic fungal tree of life (James et al. 2006), is schematic and the branch lengths are not meaningful. ATP9mt denotes a mitochondrially encoded \textit{Atp9} sequence, ATP9-5 and ATP9-7 denote homologs of \textit{PaATP9-5} and \textit{PaATP9-7}, respectively. See text for details.
**FIG. 2.** Schematic representation of *Podospora anserina* life cycle and perithecia developed from wild-type or *Δ7* female organs. (A) The *P. anserina* life cycle includes two phases: vegetative growth and sexual reproduction. The ascospore germination process (germination stage) is a critical step initiating growth to yield a mycelium, which is composed of a complex network of interconnected cells. After 48 h of germination on G medium (G48), small pieces of mycelium were transferred to M2 medium to continue the life cycle (growth stage). In standard culture conditions, the fungus displays a linear growth rate of 0.7 cm per day. On the schematic view of fungus growth in an M2 Petri dish, the arrow indicates the direction of mycelium extension; I, inoculation area; A, apical growing zone; B, C, and D, mature zones (Materials and Methods). During the growth stage, female (large ascogonia) and male (small conidia) gametes both differentiated from the same mycelium. However, fertilization occurs only between gametes of opposite mating type so that a strain can be either used as a female or a male. After fertilization, the female organs develop into fruiting bodies (perithecia, see [B]), which are the sites of caryogamy, meiosis, and asci and ascospore formation. After 96 h of fertilization, mature ascospores begin to be ejected from mature perithecia: this is the completion of one cycle. Times points chosen for RT-qPCR analyses (fig. 3) are indicated along the life cycle (G0, G8–G48; D, C, B, A; R0, R12–R96). Numberings correspond to time in hours. The scheme is not to scale. (B) Optical and microscopic views of perithecia resulting from wild-type (views 1 and 2) or *Δ7* (views 3 and 4) homozygote crosses. Pigmented structures on views 1 and 3 are representative of perithecia developed from mycelium from wild-type or *Δ7* female organs 96 h after fertilization, respectively. Views 2 and 4: rosettes of asci representative of the content of wild-type or *Δ7* mature perithecium, respectively. Perithecia were collected 96 h after fertilization and examined under the microscope. Wild-type fruiting bodies...
mature ascospores began to be ejected (fig. 3C, R96) and the two PaAtp9-5 genes were similarly strongly expressed. The increase in PaAtp9-5 transcript abundance was between 24 and 48 h after fertilization and continued until 96 h. This is fully consistent with the accumulation of PaAtp9-5 transcripts in mature ascospores (fig. 3A; G0). The stronger expression of PaAtp9-7 during sexual reproduction agrees with the failure of the Δ7 strain to produce ascospores.

The expression patterns of the PaAtp9-5 and PaAtp9-7 genes differed through the P. anserina life cycle (fig. 3D). PaAtp9-5 mRNA was abundant during germination but less so during growth and sexual reproduction. By contrast, PaAtp9-7 transcripts were nearly undetectable during germination but prominent during growth and sexual reproduction. PaAtp9 transcripts were most abundant during ascospore germination (germination stage), which is critical to trigger P. anserina life cycle.

The PaAtp9-5 and PaAtp9-7 Genes Encode Functionally Interchangeable Polypeptides

The putative mature sequences of the PaAtp9-5 and PaAtp9-7 polypeptides share only 66% identity. It is therefore possible that PaAtp9-5 and PaAtp9-7 do not encode equivalent subunits c of the mitochondrial ATP synthase. We constructed two chimeric transgenes: PaAtp9-7 CDS flanked by 5′ (585 bp) and 3′ (739 bp) regions of PaAtp9-5 (75 transgene) and PaAtp9-5 CDS flanked by 5′ (1,456 bp) and 3′ (1,403 bp) regions of PaAtp9-7 (57 transgene). The expression of these chimeric transgenes was verified by RT-qPCR (supplementary materials and methods, Supplementary Material online). The transgenes were introduced into various genetic backgrounds and ascospore germination and ascospore production processes were studied (table 2).

First, presence of the 75 transgene rescued the failure of Δ5 ascospore to initiate germination (table 2, compare row 2 with rows 4 and 8). On the contrary, the presence of the 57 transgene did not complement the Δ5 (rows 9 and 10). Thus, driving expression of PaAtp9-7 by 5′ and 3′ flanking regions of PaAtp9-5 is sufficient to allow initiation of germination. Second, the defect in ascospore production associated with the PaAtp9-7 deletion (Δ7) was efficiently rescued by the expression of the 57 transgene (table 2, compare rows 5 and 3). So, reciprocally, driving expression of PaAtp9-5 by 5′ and 3′ flanking regions of PaAtp9-7 is sufficient to ensure ascospore production, although not as well when the PaAtp9-7 polypeptide is present.

Surprisingly, the 75 transgene also improved ascospore production in the Δ7 context albeit to a lesser extent (about 30% of the wild type; table 2, rows 7 and 8). The expression of the PaAtp9-5 gene during sexual reproduction is weak (fig. 3C), so this finding was unexpected. RT-qPCR analyses showed that 75 transgene expression was about double PaAtp9-5 expression (data not shown), suggesting that a moderate increase in PaAtp9 gene expression may be sufficient to improve ascospore production significantly.

In the Δ5 Δ7 context, the expression of 57 and 75 chimeric transgenes gave rise to wild-type ascospore germination and wild-type ascospore production (table 2, row 6), evidence that the PaAtp9 coding sequences are functionally interchangeable. These various experiments show that specific transcriptional regulation of the PaAtp9-5 and PaAtp9-7 genes is required for the normal progression of the P. anserina life cycle. In addition, PaAtp9-5 and PaAtp9-7 genes encode functionally interchangeable subunits c demonstrating that they are both bona fide components of the P. anserina mitochondrial ATP synthase.

Discussion

Many fungal species contain only one Atp9 gene encoding the subunit c of ATP synthase in the mt genome. Here, we report two functional Atp9 genes (named PaAtp9-7 and PaAtp9-5) in the nuclear genome of P. anserina. There are also two functional Atp9 genes in the filamentous fungus N. crassa, but one is on the mt genome and one in the nuclear genome (van den Boogaart et al. 1982; Bittner-Eddy et al. 1994). We looked at the Atp9 gene distribution in 26 species representative of fungi, and for which the entire genome sequences are available. In addition to the situation in P. anserina and close relatives Cha. globosum and M. grisea, (e.g., two nuclear Atp9 genes with no mt Atp9 gene), we found two novel Atp9 gene distributions in the Pezizomycotina subphylum to which P. anserina and N. crassa belong: 1) two nuclear Atp9 genes coexisting with one mt Atp9 gene in Myc. graminicola, and 2) one nuclear Atp9 gene with no mt Atp9 gene, in both Pyr. tritici-repentis and Sta. nodorum.

Our phylogenetic analyses of 36 fungal Atp9 genes clearly defined two subgroups for nuclear genes (Atp9-5 or Atp9-7 like) that are both distinct from mt genes. The two Atp9 genes of P. anserina and all species with two nuclear Atp9 genes fall into the two nuclear subgroups. The polypeptides encoded by the Atp9-5 and Atp9-7-like genes have different putative MTS. Therefore, the Atp9-7 and Atp9-5-like genes presumably result from two independent gene transfers from the mitochondrion rather than from a duplication event in the nuclear genome (fig. 1D).

Position of the branches containing the polypeptides encoded by the nuclear Atp9-5 and Atp9-7-like genes in the tree (fig. 1C) suggests that they were both transferred to the nuclear genome at an early stage of the evolution of filamentous fungi. Presence of both an Atp9-5 and an Atp9-7 homolog in the genome of the last common ancestor of...
Sordariomycetes and Dothideomycetes, which would be close to the last common ancestor of all Pezizomycotina according to the six-gene phylogeny of James et al. (2006), seems to be the most parsimonious explanation (excluding scenarios involving horizontal gene transfer [HGT], discussed below) for the observed distribution of these sequences in modern fungi.

The lack of Atp9-7-like sequences in Eurotiomycetes is most likely a secondary trait resulting from the loss of the relevant gene in this lineage (fig. 1D). Generally, the

**Table 2. Genotype–Phenotype Relationships.**

<table>
<thead>
<tr>
<th>Row</th>
<th>Locus PaAtp9-5</th>
<th>Locus PaAtp9-7</th>
<th>Transgene</th>
<th>Ascospore Germination</th>
<th>Ascospore Production</th>
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<td>+</td>
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<td>Δ5 Δ7</td>
<td>+</td>
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<tr>
<td>10</td>
<td>Δ15</td>
<td>Δ17</td>
<td>Δ7</td>
<td>—</td>
<td>nt</td>
</tr>
</tbody>
</table>

*Note.—Δ5 and Δ7: deletion of the PaAtp9-5 and PaAtp9-7 gene, respectively. nt: could not be tested; +, no defect at the germination stage; −, absence of ascospore germination after ten days of observation.

*Transgenes are reported as follows: the number refers to the CDS and the superscript number to the origin of 5′ and 3′ flanking regions.

*Ascospore production efficiency estimated for homozygous crosses by counting the number of asci ejected in 15–60 min, twice a day for 4 days; +++, +, and + correspond to 85–100%, 60–80%, and 20–40% of the wild-type ascus production, respectively; −, no or few asci ejected.*
transfer of a functional gene from the organelle to the nuclear genome, with the required acquisition of a targeting sequence and regulatory elements, has to be a rare event occurring with a much lower probability than gene loss, particularly for genes with at least partially redundant functions. Transfer of a functional gene from the nucleus to the mitochondrial genome is even less likely, with no proof of it occurring naturally whatsoever. This means that any parsimonious scenario explaining the observed distribution of nuclear and mitochondrial Atp9 genes has to assume that the presence of either Atp9-5-like, Atp9-7-like, or mitochondrial Atp9 genes is ancestral, whereas the lack of any of these sequences can be due to independent gene loss events. With the mtDNA-encoded Atp9 still present, either nuclear gene (or both of them) can be lost (as in the case of Onygenales). A single nuclear Atp9-7-like gene is sufficient to support the respiratory function, and Atp9-5 and/or mitochondrial Atp9 can be lost (as in Pleosporales, where both are absent). No example of a genome with a nuclear Atp9-5 homolog being the only subunit c gene was found.

An alternative scenario would have to involve HGT to maintain consistency with the fungal tree of life phylogeny. The presence of Atp9-7 genes in some Sordariales (P. anserina, Cha. globosum, and M. grisea) could be due to an HGT event, and HGT in filamentous fungi is well documented (Mallet et al. 2010). The three members of Sordariales that contain an Atp9-7-like sequence do not, however, form a clade in the phylogeny described by James et al. (2006), and the hypothetical HGT to the ancestor of these taxa would still have to be followed by a loss of the gene from the clade containing N. crassa and S. macrospora. The advantage in parsimony of the HGT scenario over the multiple-gene loss scenario is not therefore significant, and multiple independent gene loss events need to be postulated even when HGT is considered.

Functional gene transfer to the nucleus implies that the genes gained regulatory elements for appropriate expression and, most often, a targeting sequence. We show that expression of the PaAtp9-5 and PaAtp9-7 nuclear genes is highly regulated along the life cycle of P. anserina and that the regulation of transcription was coherent with the requirement for the genes during fungal development. The PaAtp9-5 gene was massively expressed at the germination stage but was weakly expressed during growth and sexual reproduction. The PaAtp9-7 gene was prominently expressed during sexual reproduction and was probably not expressed during the ascospore germination process. The loss of PaAtp9-5 prevented ascospore germination, and deletion of PaAtp9-7 strongly impaired ascospore production. Thus, both P. anserina genes play important roles in fungal development indicating that during evolution they have acquired necessary, but nonredundant, elements to supplant the ancestor mitochondrial gene and to diverge.

The ATP9-5 and ATP9-7 polypeptide sequences are not identical, so it is plausible that the corresponding subunits c possess different functional features. However, when the flanking regions were switched between genes, the whole developmental process of the fungus was quite normal; this demonstrates that the expression levels of these genes are more important than any differences in their polypeptide sequences. It also suggests that the MTS of the ATP9-5 and ATP9-7 polypeptides both allow efficient mitochondrial import. Nevertheless, when expressed under the control of PaAtp9-7 flanking regions, ATP9-5 alone was unable to promote a wild-type level of ascospore production. In addition, a small amount of ATP9-7 appears sufficient to ensure an ascospore production suggesting some specificity of the ATP9-7 polypeptide at this stage of P. anserina development.

In mammals, the subunit c is produced from nuclear iso-genes (P1, P2, and P3) that encode polypeptides differing only by their MTS (De Grassi et al. 2006). Only expression of the P1 gene appears to be actively regulated at the transcriptional level in response to various physiological stimuli; such regulation has been described in brown adipose tissue (BAT), which is a specialized thermogenic organ (Houšek et al. 1995; Andersson et al. 1997). Upon cold acclimation, the expression of P1 is tenfold downregulated in BAT, leading to a major decrease in the amount of mitochondrial ATP synthase (Andersson et al. 1997). The other subunits of ATP synthase continue to be synthesized at the same rate, which results in their overproduction relative to subunit c and ultimately to their degradation. Thus, it is the production of subunit c (via the control of P1 expression) that determines the amount of mitochondrial ATP synthase in mammals (Kramarova et al. 2008). Our studies show that the highest level of P. anserina Atp9 transcripts (mainly due by the expression of PaAtp9-5) is during ascospore germination, a process involving substantial biomass production, and hence with large energy demands. Thus, transcriptional controls of PaAtp9-5 (and PaAtp9-7) expression may similarly modulate mitochondrial ATP synthase production in this fungus. Preliminary results from a whole-genome microarray approach comparing late and early growing stages (equivalent to zones B and A, respectively; fig. 3C) are consistent with the PaAtp9-5 and PaAtp9-7 expression patterns indicated by RT-qPCR, and suggest that the other genes (mitochondrial or nuclear) encoding subunits of the P. anserina ATP synthase are not expressed differently in these two conditions (Bidard F and Debuchy R, personal communication). Although the nuclear relocation of Atp9 genes occurred independently in fungi and mammals, this event has in both cases, been followed by the establishment of complex transcriptional regulation. This regulation of the Atp9 gene may determine the production of mitochondrial ATP synthase and thereby provide benefits to development. This evolutionary convergence may reflect substantial constraints on the assembly of the ATP synthase making Atp9 the most likely choice as the subunit for regulating the whole enzyme in mitochondria.

Our findings are also pertinent to the enigmatic question of the retention of genes in mitochondria. One view is that transfer of genes to the nucleus is active (as exemplified here for the Atp9 gene in the Pezizomycotina subphylum) and
still underway (see Adams and Palmer 2003 for a review). Alternatively, genes may have been trapped in organelles because they encode highly hydrophobic proteins that might be difficult to transport back to mitochondria for appropriate assembly or because their products are toxic if present in the cytosol. Another view, known as the CORR (colocation for redox regulation of gene expression) hypothesis, is that genes may have been preferentially maintained in organelles to ensure optimal energy transduction activity (Race et al. 1999; Allen 2003). Our observations indicate that the nuclear relocation of an mitochondrial gene may be exploited so as to allow implementation of specific regulatory mechanisms involved in development and cell differentiation. We recently found evidence (Bietenhader M, Marcos A, Tetaud E, Aiyar RS, Kucharczyk R, Godard F, Salin B, Sagot I, Gagneur J, Dequard-Chablat M, Contamine V, Selmém CH, Hermann-Le Denmat S, Annie Sainsard-Chanet A, Steinmetz LM, di Rago JP, in preparation) that in the yeast S. cerevisiae the transfer of the mitochondrial Atp9 gene to the nucleus is a very complex process: it appears to have required a number of cellular accommodations in addition to gene recoding and mitochondrial-targeting information, so as to optimize expression of the gene from the nucleus. Thus, if transfer of the Atp9 gene to the nucleus does not confer any significant advantage to the cell, the gene is likely to stay within the organelle. Clearly, the presence of Atp9 in the nucleus of P. anserina is exploited to modulate energy metabolism throughout the life cycle of the fungus; this may explain why despite the difficulties of transferring this gene to the nucleus, it finally succeeded in escaping from the organelle.

Supplementary Material
Supplementary figure S1 and tables S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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