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The Crucial Role of the Pls1 Tetraspanin during Ascospore Germination in *Podospora anserina* Provides an Example of the Convergent Evolution of Morphogenetic Processes in Fungal Plant Pathogens and Saprobes▽†

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Pls1 tetraspanins were shown for some pathogenic fungi to be essential for appressorium-mediated penetration into their host plants. We show here that *Podospora anserina*, a saprobic fungus lacking appressorium, contains *PaPls1*, a gene orthologous to known *PLS1* genes. Inactivation of *PaPls1* demonstrates that this gene is specifically required for the germination of ascospores in *P. anserina*. These ascospores are heavily melanized cells that germinate under inducing conditions through a specific pore. On the contrary, *MgPLS1*, which fully complements a Δ*PaPls1* ascospore germination defect, has no role in the germination of *Magnaporthe grisea* nonmelanized ascospores but is required for the formation of the penetration peg at the pore of its melanized appressorium. *P. anserina* mutants with mutation of *PaNox2*, which encodes the NADPH oxidase of the NOX2 family, display the same ascospore-specific germination defect as the Δ*PaPls1* mutant. Both mutant phenotypes are suppressed by the inhibition of melanin biosynthesis, suggesting that they are involved in the same cellular process required for the germination of *P. anserina* melanized ascospores. The analysis of the distribution of *PLS1* and *NOX2* genes in fungal genomes shows that they are either both present or both absent. These results indicate that the germination of *P. anserina* ascospores and the formation of the *M. grisea* appressorium penetration peg use the same molecular machinery that includes Pls1 and Nox2. This machinery is specifically required for the emergence of polarized hyphae from reinforced structures such as appressoria and ascospores. Its recurrent recruitment during fungal evolution may account for some of the morphogenetic convergence observed in fungi.

Convergent evolution of trophic strategies and morphogenetic processes is a hallmark of fungi (27). For example, many fungi belonging to different fungal clades are phytopathogenic, whereas their close relatives are saprobic. These plant parasitic fungi may penetrate into their host plants using a specialized structure called the appressorium. Appressoria are attached to the surface of plants and redirect fungal growth into the host tissues underneath through the formation of a penetration peg. Due to their importance in pathogenicity, appressoria are studied in fungi such as *Magnaporthe grisea*, a devastating pathogen of rice (25, 54), *Botrytis cinerea*, a wide-host-range plant pathogen (19), and *Colletotrichum lindemuthianum*, an important pathogen of beans (13). These three fungi belong to two different clades of ascomycetes. *M. grisea* and *C. lindemuthianum* are in the *Sordariomycetes* and *B. cinerea* in *Leotiomycetes*. A significant number of species related to these three fungi are saprobic. Which of the parasitic or saprobic lifestyles displayed by these organisms arise by convergent evolution is thus crucial to understand their evolution. An answer to this question is to establish whether appressoria are homoplasious or homologous in *Sordariomycetes* and *Leotiomycetes* and whether appressorium-specific processes may also be found in saprobic fungi.

Appressoria of *M. grisea*, *C. lindemuthianum*, and *B. cinerea*, have different morphologies, cellular organizations, and ontologies. In *M. grisea* and *C. lindemuthianum*, appressoria are differentiated at the tip of germ tubes issued from asexual spores on contact with a plant surface. These appressoria are dome-shaped single cells that are heavily melanized. It has been shown that collapse of the spore and transfer of its content to the appressorium are necessary for functionality in *M. grisea* (49). In *B. cinerea*, appressoria are also differentiated at the tip of germ tubes issued from asexual spores on contact with a plant surface. However, this structure is also called a pseudo-appressorium because it is not an individualized cell. Indeed, *B. cinerea* pseudo-appressoria are still connected to the spore from which they are derived without the formation of a septum. Furthermore, they are not heavily melanized, although they display a reinforcement of their cell wall (19). This

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observation suggests that these two types of appressoria have evolved convergently: i.e., appressoria from *Leotiomycetes* and *Sordariomycetes* are homoplasious. Alternatively, they could be homologous structures despite these morphogenetic differences. In support of the homology hypothesis, it has been shown that appressoria from these three fungi require a functional tetraspanin gene to infect host plants (11, 19, 51). Indeed *Mgpls1*$^-$, *Clpls1*$^-$, and *Bcpls1*$^-$ deletion mutants are nonpathogenic. They are all specifically impaired in the production of the penetration peg, while their appressoria appear normal. These data suggest an evolutionary conservation of appressorium physiology among these fungi and therefore homology of appressoria instead of homoplasy. However, the recurrent use of the Pls1 tetraspanins during fungal evolution for a function specific to appressoria cannot be ruled out.

Tetraspanins are eukaryotic small integral membrane proteins identified in animals and fungi (26, 50). At least 33 distinct tetraspanins were identified in humans, 37 in *Drosophila melanogaster*, and 20 in *Caenorhabditis elegans*, but only 3 in fungi: Pls1 and Tps2 in basidiomycetes and Pls1 and Tps3 in ascomycetes (26, 31). In *M. grisea*, Pls1 is expressed in the appressoria and localizes either at the plasma membrane or in vacuoles (11), while it is expressed in both appressoria and vegetative mycelium in *C. lindemuthianum* (51) and in conidia, germ tubes, and appressoria during host penetration in *B. cinerea* (19). Although these proteins display limited sequence similarities, they share conserved secondary structures including four transmembrane domains and a cysteine based pattern in their EC2 large extracellular loop (42, 48). In animals, tetraspanins act as molecular adaptors involved in the formation of protein complexes localized in tetraspanin-enriched microdomains of membranes (23), called the "tetraspanin web" (7). Animal tetraspanins are involved in different biological processes including sperm-egg fusion, entry of parasites or viruses into host cells, cell-cell interactions in central nervous system or immune system (B-cell–T-cell immunological synapse), cell adhesion, motility, polarity, and trafficking of membrane proteins (23, 33).

Surprisingly, additional Pls1 orthologues were identified in saprobic fungi devoid of appressorium such as *Neurospora crassa* (20), *Coprinopsis cinerea* (26), *Chaetomium globosum*, *Podospora anserina*, *Trichoderma reesei*, and *Phanerochete chrysosporium* (31). To date, the role of Pls1 tetraspanins in these nonpathogenic fungi is unknown. In this report, we used the model ascomycete *Podospora anserina*, a coprophilous fungus closely related to *N. crassa*, to study the role of Pls1 in a saprobic fungus devoid of appressorium. The phenotype of the *PaPls1* deletion mutant revealed a crucial role for this gene in germination of ascospores, which are heavily melanized in this species. Interestingly, the germination of nonmelanized *M. grisea* ascospores is unaffected in the *MgPLS1* deletion mutant. These data suggest that the germination of *P. anserina* melanized ascospores and the formation of the penetration peg in *M. grisea* melanized appressoria share homologous determinants. Our data thus provide new insights indicating that the ability to generate a peg through a pore of a reinforced structure (including appressoria and ascospores) may be gained by convergent evolution through the recruitment of a highly specialized homologous machinery, providing a strong argument for the recurrent convergent evolution of morphogenetic processes in fungi.

MATERIALS AND METHODS

Strains and culture conditions. The *P. anserina* S (big S) strain (39) used for this study has its genome sequence available at http://podospora.igmors.u-psud.fr (17). The *pks1-193* (formerly *193*) *P. anserina* big S strain carries a mutation in a polyketide synthase gene required for melanin biosynthesis. It displays nonpigmented mycelia, perithecia, and ascospores (12, 38). Standard culture conditions, media, and genetic methods were described previously (40). Menadione was added at 10^{-4} to 10^{-6} M, *t*-butyl-hydroxyperoxide at 10^{-5} to 10^{-7} M, and H_2O_2 at 0.25, 0.05, and 0.01%. *P. anserina* is pseudohomothallic, producing homokaryotic and heterokaryotic mycelia from small and large ascospores, respectively.

An s (small s) Δ*mus51*::*phleoR* strain was kindly provided by C. Sellem. It has undergone deletion of its *mus51* gene, encoding the Ku70 protein involved in nonhomologous end-joining DNA repair, and has an increased frequency of targeted gene replacement (16). The Δ*mus51*::*phleoR* mutation was introduced in the S strain by 10 successive backcrosses. The phleomycin marker of the Δ*mus51*::*phleoR* big S mutant was replaced by the *su8-1* marker using homologous recombination, and Δ*mus51*::*su8-1* big S strains of both mating types were constructed. These Δ*mus51*::*su8-1* strains also displayed a high frequency of targeted gene replacement (see below). Δ*mus51*::*su8-1* strains were crossed with *pks1-193* mutants of the opposite mating type to recover *pks1-193* Δ*mus51*::*su8-1* *mat*$^+$ and *pks1-193* Δ*mus51*::*su8-1* *mat*$^-$ progeny.

DNA manipulation and deletion of *P. anserina PaPls1* by targeted gene replacement. DNA was extracted as described previously (32), and standard protocols were followed for DNA manipulation (2). The upstream (LB [700 bp]) and downstream (RB [750 bp]) regions of *PaPls1* were obtained by PCR amplification using *P. anserina* big S strain genomic DNA as a template and primer pairs Pls1-L1/Pls1-L2-SfiIa and Pls1-R1-SfiIb/Pls1-R2, respectively (see Table S1 in the supplemental material). The hygromycin B resistance cassette (*hph*) was obtained by digesting the plasmid pFV8 (52) with SfiI (hph/SfiI). The amplified 700-bp LB and 750-bp RB fragments from the *PaPls1* locus were digested with SfiI and ligated with T4 DNA ligase (Roche) to an hph/SfiI fragment. The amplification of the 2.8-kb *PaPls1* replacement cassette was obtained using primers Pls1-L3 and Pls1-R3 and the previous ligation product as a template. This final PCR fragment was used to transform *P. anserina* protoplasts from *pks1-193* Δ*mus51*::*su8-1 mat*$^+$ and *pks1-193* Δ*mus51*::*su8-1 mat*$^-$ strains as described previously (9). Three hygromycin B-resistant transformants (hygR) were obtained, one *mat*$^-$ and two *mat*$^+$. These primary transformants were crossed to a wild-type strain, and progeny with pigmented ascospores lacking *pks1-193* or *pks1-193* Δ*mus51*::*su8-1* mutations were recovered. Δ*PaPls1*::*hph* mutants were distinguished from Δ*mus51*::*su8-1* Δ*PaPls1*::*hph* mutants by the ability of *su8-1* to suppress of the lack of pigmentation of *pks1-193* ascospores in additional crosses with a *pks1-193* mutant. Progeny lacking *pks1-193* and Δ*mus51*::*su8-1* were obtained, and the replacements at the *PaPls1* locus were assessed by Southern hybridization as depicted in Fig. S1 in the supplemental material. Two *PaPls1* deletion mutants, one *mat*$^+$ and one *mat*$^-$, were selected for further studies.

Complementation of *PaPls1* deletion mutants with wild-type *PaPls1* and phenotypic analyses. Plasmid pCM421-8.5 carries the *M. grisea MgPLS1* wild-type allele (11). Green fluorescent protein (GFP) was fused to the C terminus of MgPLS1 under the control of *MgPls1* promoter and terminator. NcoI and XbaI sites were introduced by PCR just before the Stop codon of *MgPls1* by using a 937-bp subclone from pCM421-8.5 as a template. pEGFP (Clontech, Ozyme) was used to obtain a PCR product containing an NcoI site at the start of the GFP open reading frame (ORF) and a XbaI site at the end of the GFP ORF. This PCR product was fused just before the Stop codon of the modified *MgPls1*–937-bp NruI subclone and reintroduced as an NruI fragment into pCM421-8.5 to obtain the plasmid pCM421-8.5-*MgPls1*-GFP. This plasmid and the pBC-phleo plasmid conferring resistance to phleomycin (43) were used to cotransform the Δ*PaPls1 mat*$^-$ mutant.

P. anserina life span and crippled growth phenotypes were assessed as previously described (45, 46). Hyphal interference, peroxide accumulation, and cell death at mycelial confrontation zones was assayed as described previously (44). Perithecium formation, fertility in crosses, and ascospore germination were assessed as described previously (36). *M. grisea* crosses were performed as described previously (47). Four *Mgpls1*::*hph* progenies from the cross between the original punchless mutant (*Mgpls1*::*hph*) (11) and a wild-type *M. grisea* strain (M4) of the opposite mating type were used to perform two wild-type × *Mgpls1*::*hph* and two *Mgpls1*::*hph* × *Mgpls1*::*hph* crosses. Eighteen days after mycelium confrontation, three perithecia from each cross were deposited on

water agar (4.5% agar) and opened with a sterile scalpel under a stereoscopic binocular to liberate asci. After 16 h on water agar at 26°C, ascospore germination was observed.

Wounded barley leaf fragments (cv. Plaisant) were inoculated with 50-μl droplets of spore suspension (3.10^5 spores/ml) from *M. grisea* wild-type strain P1.2 or the Δ*Mgpls1* P1.2 mutant as described previously (18). Tricyclazole stock solution (Ehrenstorfer GmbH, Augsburg, Germany) was mixed with spores at a final concentration of 20 μg/ml before inoculation.

Phylogenetic analysis. Protein sequences were aligned using ClustalX 1.8 and transferred to GenDoc for visualization. This alignment was used to construct a phylogenetic tree using the maximum likelihood method (PHYML software) (21, 22) and transferred to Mega3.1 for visualization (30). Bootstrap values are expressed as percentages of 100 replicates.

RESULTS

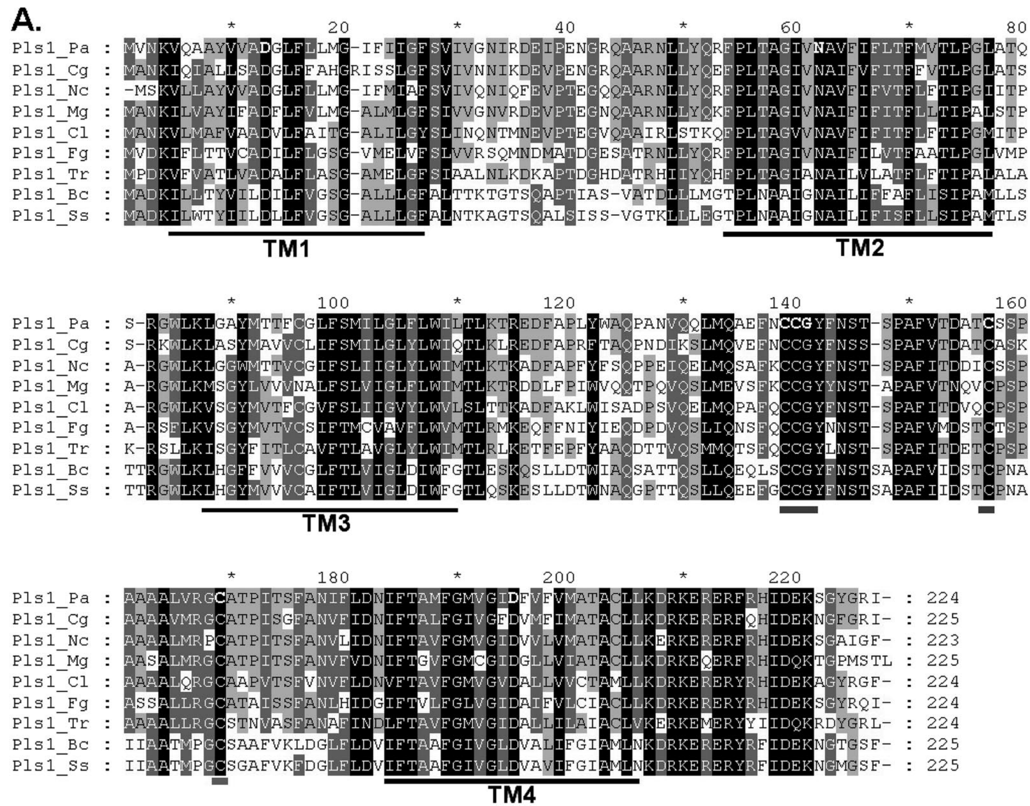
Identification of the Pls1 tetraspanin-encoding gene from *P. anserina*. A single MgPls1 hit (Pa_1_19270; E value, e−76) was identified in the *P. anserina* predicted proteome (http://podospora.igmors.u-psud.fr) (17) using BlastP (1) and designated as *PaPls1*. The PaPls1 protein sequence displayed 65% identity and 83% similarity to MgPls1 (Fig. 1A). *PaPls1* is located on *P. anserina* chromosome 1 close to the mating-type locus in a region devoid of recombination. Eighteen *PaPls1* expressed sequence tags (ESTs) were identified (*P. anserina* EST database) (17), including 13 ESTs from mycelia, 4 from developing fructifications (perithecia), and 1 from germinating ascospores. The recovery of *PaPls1* ESTs obtained from different tissues suggests that this gene is transcribed throughout the life cycle of the fungus. These ESTs showed that the exon/intron boundaries predicted in Pa_1_19270 were correct. *PaPls1* displays two exons of 402 bp and 273 bp, interrupted by an intron of 76 bp. *MgPLS1* has an additional intron (20).

Analysis of the PaPls1 protein sequence with *TMHMM2.0* (http://www.cbs.dtu.dk/services/TMHMM-2.0/) revealed four transmembrane segments (TM1 to -4) that delimit a small extracellular loop (EC1), a small intracellular loop (IC), a large extracellular loop (EC2), and a short cytoplasmic tail similar in size and locations (Fig. 1A) as Pls1 tetraspanins (20). PaPls1 TMs have charged/polar amino acids conserved among fungal tetraspanins, and PaPls1 ECL2 displayed the same cysteine-based pattern as Pls1 tetraspanins (CCG-X_{14}-C-X_{10}-C [Fig. 1A]). Finally, the C-terminal tail of PaPls1 is almost identical (>70% identity [Fig. 1A]) to the conserved C-terminal tail sequence of Pls1 tetraspanins (20). This analysis shows that PaPls1 has all the structural hallmarks of Pls1 tetraspanins (20, 31, 50), suggesting that it corresponds to a functional protein.

Fungal Pls1 tetraspanins identified in the ascomycetes *B. cinerea*, *C. lindemuthianum*, *N. crassa*, and *Gibberella zeae* (20, 26, 50) were aligned with PaPls1. The CgPls1 tetraspanin was identified in the genome of *Chaetomium globosum* (CHGG_06472.1 at http://www.broad.mit.edu/annotation/genome/chaetomium_globosum/Home.html), and its protein sequence was aligned with those of Pls1 tetraspanins. This alignment was used to construct a phylogenetic tree using the maximum likelihood method (PHYML) (22). This phylogenetic tree (Fig. 1B) shows that PaPls1 is highly similar to CgPls1, as expected since *C. globosum* is a fungal species closely related to *P. anserina* (27). The position of the single *PaPls1* intron that is conserved among *PLS1* genes from pyrenomycetes (20) and the topology of the phylogenetic tree suggest that Pls1 tetraspanins are encoded by a family of orthologous genes.

Inactivation of *PaPls1* leads to a strong defect in ascospore germination. To investigate the role of PaPls1 in the *P. anserina* life cycle, we constructed a deletion mutant (Δ*PaPls1*) by targeted gene replacement. As *PaPls1* is closely linked to the mating-type locus in a region devoid of recombination, we constructed Δ*PaPls1* deletion mutants in *pks1-193* Δ*mus51*::*su8-1* strains with different mating types. These Δ*PaPls1* mutants of opposite mating types are needed to study the role of PaPls1 in the sexual cycle that requires crosses between Δ*PaPls1* mutants. Transformants carrying only the Δ*PaPls1* deletion were obtained by crossing and characterized by Southern hybridization (see Fig. S1 in the supplemental material). This analysis revealed that the *PaPls1* gene was replaced by the *hph* resistance cassette in different transformants designated as Δ*PaPls1 mat*$^+$ and Δ*PaPls1 mat*$^-$, respectively. Δ*PaPls1* mutants are identical to the wild type for vegetative phenotypes, including growth rate, mycelium morphology, life span (37), ability to display crippled growth cell degeneration (45), and hyphal interference (44). Δ*PaPls1* mutants were also tested for their ability to perform cell fusion after hyphal anastomosis. Δ*PaPls1 mat*$^+$ *lys2-1* and Δ*PaPls1 mat*$^+$ *leu1-1* mutants unable to grow on minimal medium were constructed by crossing the Δ*PaPls1 mat*$^+$ strain with auxotrophic mutants. Mycelia from both double mutants were mixed and deposited onto M2 minimal medium. Mycelia able to grow on minimal medium were observed as soon as 24 h after plating, at the same rate as those formed when mixing mycelia from *lys2-1* and *leu1-1* strains. These results demonstrate that hyphae from Δ*PaPls1 mat*$^+$ *lys2-1* and Δ*PaPls mat*$^+$ *leu1-1* strains are fusing and forming heterokaryotic hyphae growing on minimal medium, as observed for the wild type.

Crosses between Δ*PaPls1 mat*$^+$ and Δ*PaPls1 mat*$^-$ mutants led to normal sexual fructifications (perithecia) with mature ascospores ejected as in wild-type crosses. However, only 1 out of 10^4 Δ*PaPls1* ascospores germinated on the germination-inducing G medium, unlike wild-type ascospores, which germinated at 100% on this medium. On culture media unable to induce germination, such as M2 minimal medium, Δ*PaPls1* ascospores germinated at a frequency identical to that of the wild type (10^{-4}). The Δ*PaPls1* defect was ascospore autonomous since ascospores from wild-type progeny obtained in wild-type × Δ*PaPls1* crosses germinated normally, whereas those from Δ*PaPls1* progeny did not. *PaPls1*$^+$/Δ*PaPls1* dikaryotic ascospores had a wild-type germination rate, showing that the Δ*PaPls1* defect was recessive. In *P. anserina*, two cells form the ascospore, a large heavily melanized one and a small hyaline one, called the primary appendage. On G medium, wild-type ascospore germination occurs at a germ pore located at the anterior side of the ascospore (i.e., at the opposite end of the primary appendage) (3, 35). The first stage of germination is the extrusion of a spherical structure from the pore that reaches up to one-third of the size of the ascospore (Fig. 2), called the "germination peg." This extrusion starts a few hours after the deposition of ascospores on the inducing medium (usually between 4 and 10 h, depending on the batch of germination medium) and is completed in 10 to 15 min (Fig. 2). Immediately after this extrusion, new polarized hyphae originate from the germination peg within a few minutes and



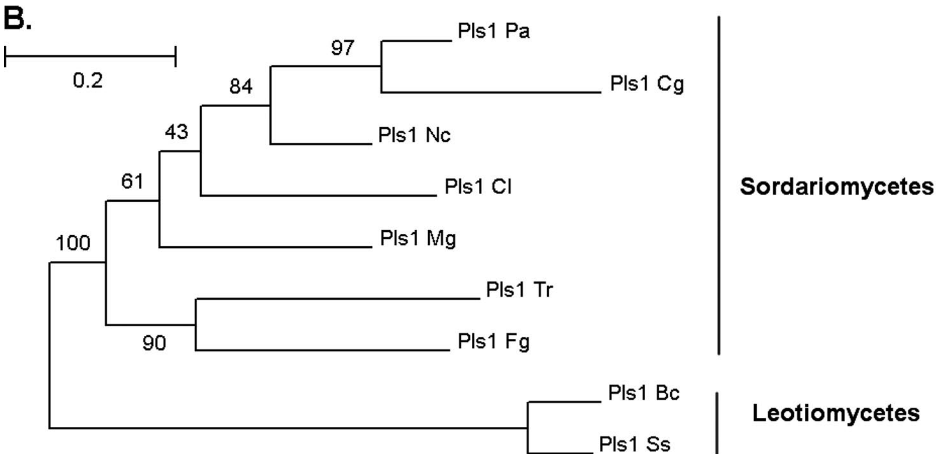


FIG. 1. Alignment and phylogenetic tree of *Sordariomycetes* and *Leotiomycetes* Pls1 tetraspanins. (A) Pls1 proteins from *Sordariomycetes* and *Leotiomycetes* display numerous conserved motifs in transmembrane domains (TM1 to -4 [underlined in black]), in ECL2 including a cysteine pattern (underlined in gray), and in the C-terminus tail. Protein sequences were aligned using ClustalX 1.8. Conserved amino acids are boxed in black (identical) and gray (similar). (B) PaPls1 clusters with CgPls1 and NcPls1, as expected, since the corresponding fungal species are closely related. The phylogenetic tree was constructed using the previous alignment (A) and a maximum likelihood method (PHYML) with BcPls1 and ScPls1 as outgroups. Bootstrap values are expressed as percentage of 100 replicates. Abbreviations for *Sordariomycetes*: Pls1_Pa, *Podospora anserina* Pa_1_19270; Pls1_Cg, *Chaetomium globosum* CHGG_06472.1; Pls1_Nc, *Neurospora crassa* AJ504996; Pls1_Cl, *Colletotrichum lindemuthianum* AJ504995; Pls1_Mg, *Magnaporthe grisea* AX058239; Pls1_Fg, *Fusarium graminearum* FG08695.1; Pls1_Tr, *Trichoderma reesei jgi Trire2 4514 fgenesh1_pm.C_scaffold_13000033*. Abbreviations for *Leotiomycetes*: Pls1_Bc, *Botrytis cinerea* AJ504994; Pls1_Ss, *Sclerotinia sclerotiorum* SS1G_05586.1.

develop into mycelia. In the Δ*PaPls1* mutants, the ascospore germination process is stopped at an early stage since germination pegs are not formed (Fig. 3).

***M. grisea MgPLS1* can functionally replace *PaPls1*.** The Δ*PaPls1 mat*$^-$ strain was cotransformed with a vector carrying the *PaPls1* wild-type allele and the pBC-phleo plasmid conferring resistance to phleomycin (43). Eight out of 45 transformants resistant to phleomycin produced ascospores that germinated with 100% efficiency when crossed with a Δ*PaPls1 mat*$^+$ strain. In the control experiment, transformation was

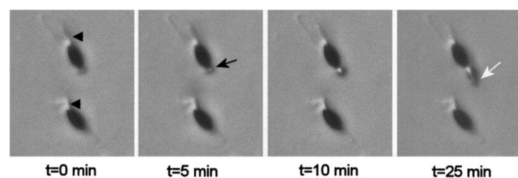


FIG. 2. Time course of the germination of a wild-type *P. anserina* ascospore on inducing medium. *P. anserina* ascospores are bicellular, with one large heavily melanized cell and a small hyaline one, the primary appendage (arrowheads). The germination starts with the extrusion of a peg (black arrow) at the pole opposite to the primary appendage, where the germ pore is located (3). The white arrow points to a newly formed hypha with polarized growth issued from the spherical germination peg. This hypha appears 20 min after the germination peg.

done with an empty vector. All of the 100 transformants obtained produced nongerminating ascospores. This result demonstrates that the ascospore germination defect of Δ*PaPls1* is due to the inactivation of *PaPls1*. The Δ*PaPls1 mat*$^-$ mutant was also cotransformed with pBC-phleo plasmid and pCM421-8.5-*MgPls1*-GFP plasmid that expressed an MgPLS1-GFP fusion protein. The transformants resistant to phleomycin were crossed with the Δ*PaPls1 mat*$^+$ mutant, and the resulting ascospores were assessed for germination on inducing medium. Two phleomycin-resistant transformants among 48 displayed a 100% ascospore germination frequency on inducing medium similar to that of the wild type. Both expressed a fluorescent protein, demonstrating that *MgPLS-GFP* complements the Δ*PaPls1* mutation. Therefore, *MgPLS1* is a functional orthologue of *PaPls1*.

The *M. grisea* ΔMgpls1 mutant has a normal ascospore germination efficiency. Since Δ*PaPls1* mutants have a strong defect in ascospore germination, we assessed the germination efficiency of asexual spores and ascospores from an *M. grisea MgPLS1* deletion mutant (Δ*Mgpls1*). Asexual spores from the Δ*Mgpls1* mutant germinated normally. The M*gPLS1* original insertion mutant (*Mgpls1*::*hph*), which displays the same pathogenicity defect as the Δ*Mgpls1* mutant, was previously crossed to wild-type *M. grisea* strain M4 (11). Their progeny were analyzed for their resistance to hygromycin B (*Mgpls1*::*hph*), pathogenicity, and mating type by performing novel *Mgpls1*::*hph* × wild-type crosses (11). The recovery of nonpathogenic progeny resistant to hygromycin (*Mgpls1*::*hph* genotype) at a frequency (45%) expected for single-gene segregation, suggests that this mutation has no effect on ascospore germination. To confirm this hypothesis, Δ*Mgpls1* progeny of opposite mating types were crossed, yielding fertile perithecia that produced ascospores with a normal germination rate of 90%. These results show that the inactivation of *MgPLS1* has no effect on ascospore germination in *M. grisea*.

Phenotypes of *PaPls1* and *PaNox2* deletion mutants are identical. The mutant with deletion of the *PaNox2* gene, which encodes an NADPH oxidase (36), is the only known *P. anserina* mutant that has the same specific recessive and autonomous defect in ascospore germination as the Δ*PaPls1* mutant. NADPH oxidases are membrane enzymes involved in the production of superoxide in a wide range of eukaryotes for signaling or defense purposes (4). Like *PaPls1*, *PaNox2* is expressed throughout the life cycle since nine ESTs for this genes are present in the database (17): six from mycelia and three from perithecia. Ascospores of the Δ*PaNox2* mutant are stopped at an early stage of their germination process, since no germination peg is formed as observed for Δ*PaPls1* ascospores (Fig. 3). Addition to the germination medium of compounds generating reactive oxygen species such as menadione (a quinone-generating superoxide in the cells), *t*-butyl-

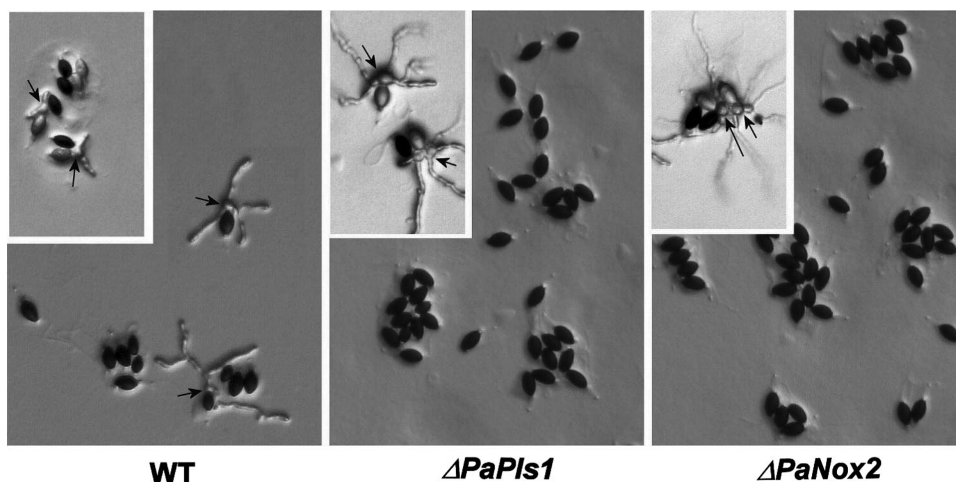


FIG. 3. Lack of *P. anserina* ascospore germination in Δ*PaPls1* and Δ*PaNox2* mutants. Germination of melanized (main panels) and unmelanized (inserts) ascospores on inducing medium. Ascospores from the main panels originate from crosses between wild type and wild type, Δ*PaPls1* and Δ*PaPls1*, and Δ*PaNox2* and Δ*PaNox2* strains of opposite mating types. Ascospores from the inserts originate from crosses between the wild type and *pks1-193* (WT), Δ*PaPls1* and *pks1-193* Δ*PaPls1* (Δ*PaPls1*), and Δ*PaNox2* and Δ*PaNox2 pks1-193* (Δ*PaNox2*) strains. Arrows point to germination pegs. In the main panels, melanized ascospores from Δ*PaPls1* and Δ*PaNox2* mutants are unable to germinate, while wild-type ascospores do germinate. In the inserts, Δ*PaPls1* and Δ*PaNox2* melanized ascospores did not germinate, while several Δ*PaPls1* and Δ*PaNox2* unmelanized ascospores did germinate, demonstrating that the inhibition of melanin biosynthesis suppresses Δ*PaPls1* and Δ*PaNox2* defects. In the wild-type × *pks1-193* cross (WT insert), only the nonmelanized ascospores have germinated when the picture was taken, indicating that ascospores lacking melanin germinate before pigmented ones.

hydroxyperoxide (an inorganic peroxide) or hydrogen peroxide failed to restore Δ*PaPls1* ascospore germination. The same result was already observed for Δ*PaNox2* ascospores (36).

We have previously shown that ascospores from the double mutant carrying the *pks1-193* and the Δ*PaNox2* mutations germinate at a high frequency (50%) on both inducing and noninducing media, like ascospores from the *pks1-193* mutant (36). These experiments show that *pks1-193* mutation suppresses the germination defect of Δ*PaNox2* ascospores. The *pks1-193* mutants carry a recessive null mutation in a *P. anserina* gene encoding the polyketide synthase involved in the first step of 1,8-dihydroxynaphthalene (DHN) melanin biosynthesis (12). These mutants are unable to synthesize DHN melanin and display unmelanized hyphae, perithecia, and ascospores. Crosses between *pks1-193* Δ*PaPls1* mat^{+} and *pks1-193* Δ*PaPls1* mat^{-} strains were performed, and the ascospores produced germinated at high frequency (50%) on both inducing and noninducing media. This result shows that the absence of melanin suppressed the Δ*PaPls1* ascospore germination defect. Unmelanized *pks1-193*, *pks1-193* Δ*PaNox2*, or *pks1-193* Δ*PaPls1* ascospores germinate rapidly after deposition onto the inducing medium. Their germination peg, which is often larger, is produced earlier than in wild-type melanized ascospores (usually 2 to 5 h after being deposited on the petri plates), although it develops in 10 to 15 min as in the wild type (Fig. 3). As in wild-type ascospores, the germination peg from these mutants switches to polarized growth and differentiated filamentous hyphae.

In *M. grisea*, the inhibition of the DHN melanin biosynthesis, either genetically in melanin-deficient mutants or chemically using inhibitors such as tricyclazole, leads to nonmelanized appressoria unable to penetrate into intact host tissues (10, 14, 24). However, *M. grisea* melanin-deficient mutants affected in the *Alb*, *Rsy*, and *Buf* genes cause lesions similar to the wild type when inoculated on wounded leaves, presumably as a consequence of their ability to penetrate through wounds. The pathogenicity defect of the Δ*Mgpls1* mutant differs strongly from that of melanin-deficient mutants as Δ*Mgpls1* mutants are unable to penetrate both unwounded and wounded leaves (11). Δ*Mgpls1* spores treated with tricyclazole differentiated unmelanized appressoria but were unable to infect intact or wounded barley leaves (no lesions [data not shown]). Therefore, the inhibition of melanin biosynthesis does not suppress the penetration and colonization defects of Δ*Mgpls1*.

Co-occurrence of *PLS1* and *NOX2* genes in fungal genomes. To further support the hypothesis that both *PLS1* and *NOX2* genes are involved in the same pathway, we have searched in available sequences of fungal genomes for the co-occurrence of these two genes. As seen in Table S2 in the supplemental material, whereas *NOX2* was found in *Batrachochytrium dendrobatidis*, a Chytridiomycota, *PLS1* seems to be present only in higher fungi (i.e., the Dikarya). Strikingly, in higher fungi, *NOX2* and *PLS1* are either both present, as in *P. anserina*, *M. grisea*, and *N. crassa*, or both absent, as in *Aspergillus* or *Mycosphaerella*. The absence of *PLS1*/*NOX2* likely results from losses in different clades of ascomycete and basidiomycete species. The absence of these two genes seems to rely on independent events since they are not located on the same chromosomal locus in fungi. The loss of *PLS1*/*NOX2* has occurred repeatedly in both ascomycete and basidiomycete species, especially in lineages with species living mostly as yeasts, such as Taphrinomycotina and Saccharomycotina yeasts and *Sporobolomyces roseus*. Fungal species that have both *PLS1* and *NOX2* differentiate either melanized appressoria or melanized ascospores germinating through a pore, while fungi lacking *PLS1*/*NOX2*, such as *Aspergillus*, *Ustilago maydis*, and *Cryptococcus neoformans*, mostly behave as yeast cells or form melanized filamentous hyphae (53, 56), without being able to differentiate reinforced structures germinating through a pore. These data strongly suggest that they have been maintained during evolution as a consequence of their involvement in the same cellular function.

DISCUSSION

Phenotype of *P. anserina* Δ*PaPls1* mutants. *P. anserina* is a fungus that lives as a saprobe on herbivore dung, does not differentiate appressoria, and does not attack plants. Nevertheless, it possesses an expressed gene coding for a tetraspanin of the Pls1 family. Thanks to its ease of handling, this fungal species is well suited to study the role of Pls1 tetraspanin in general cellular processes other than pathogenicity on plants. Most animal tetraspanins are involved in cell fusion (e.g., Cd9) (23) or in cell-cell interactions (e.g., Cd81) (33). Therefore, it was important to assess if *PaPls1* has a role in either sexual or vegetative cell fusion. Our results show that *PaPls1* is not required for sexual and vegetative cell fusion (anastomosis), as indicated by the behavior of *PaPls1* null mutants in cell fusion during sexual crosses or during the formation of vegetative heterokaryons. Δ*PaPls1* mutants also exhibited normal hyphal interference, indicating that these mutants interact normally with other fungi and were similar to the wild type with regard to mycelial growth on different media, senescence, and crippled growth cell degeneration (29, 37, 45). Following fertilization, *PaPls1* null mutants have a normal sexual process, as they matured fructifications like the wild type. However, we showed that *PaPls1* has an important role in the life cycle of *P. anserina*, as it is required for the germination of melanized ascospores. This defect is the only phenotype we observed in *PaPls1* deletion mutants.

Roles of Pls1 tetraspanins in *P. anserina* and *M. grisea*. *MgPLS1* and *PaPls1* genes are functional orthologues, since *MgPLS1* fully complements the germination defect of the Δ*PaPls1* null mutant. Yet, *MgPLS1* is required for the formation of the penetration peg originating at the pore of *M. grisea* melanized appressorium (11), while it is required for ascospore germination in *P. anserina*. We have verified that asexual spores and sexual ascospores from the *M. grisea Mgpls1* deletion mutant germinate as efficiently as the wild type, as this phenotype was not reported previously (11). Therefore, the phenotypes in both fungal species strongly differ. While *P. anserina* does not differentiate appressoria, both species produce ascospores. However, it should be noted that ascospores from these related fungal species are quite different (Fig. 4). *M. grisea* ascospores are tetracellular nonmelanized structures (28, 55), while *P. anserina* ascospores are bicellular structures with only one cell being heavily melanized (3). In *M. grisea*, ascospore germination is similar to vegetative spore germination, with germ tubes frequently originating without latency at opposite tips of a single ascospore on any media, including water

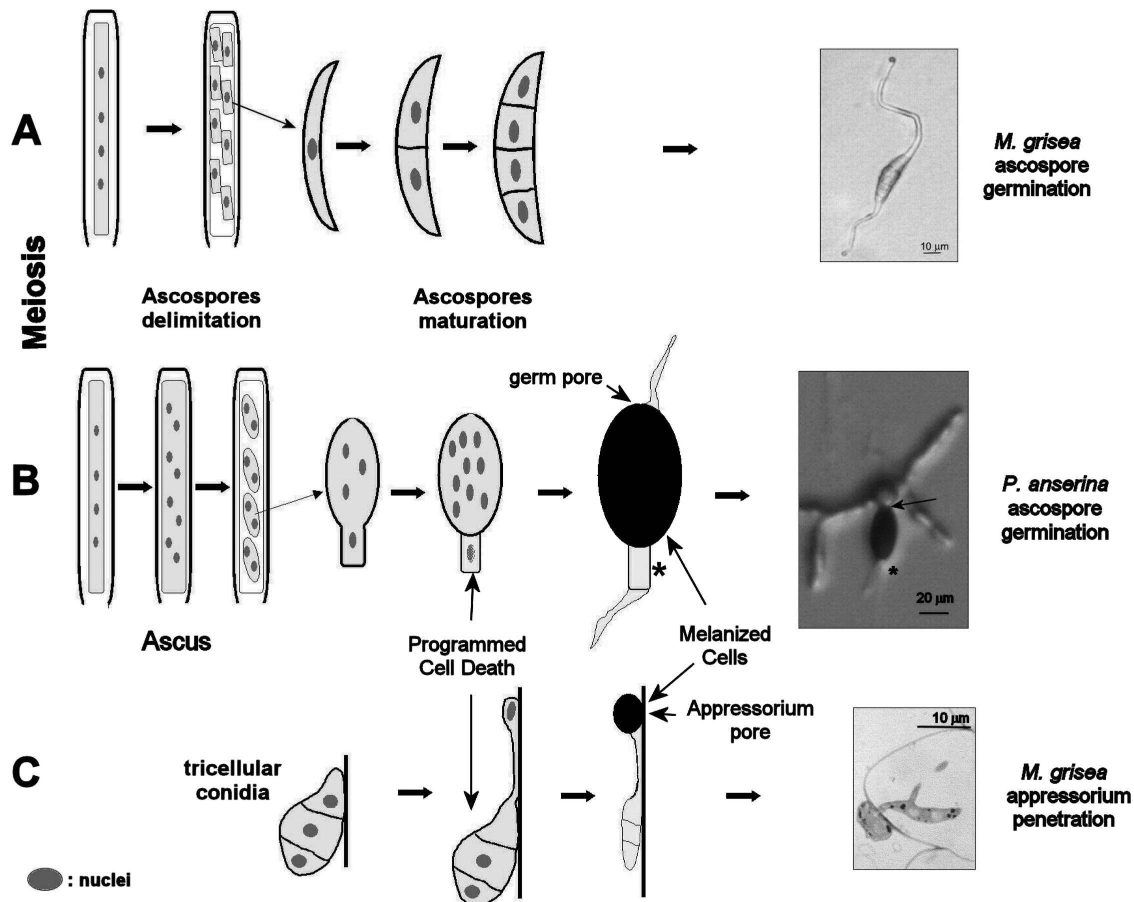


FIG. 4. Ontogeny of ascospore and appressorium in *P. anserina* and *M. grisea*. (A) Formation of ascospores in *M. grisea* results in four-cell hyaline ascospores that germinate spontaneously (55). Ascospores were observed 16 h after deposition onto water agar (4.5% agar) at 26°C. The picture shows the germination of a wild-type ascospore that occurred at two opposite tips of the spore. (B) Formation of ascospore in *P. anserina* results in two-celled ascospores (3). One cell undergoes enlargement and melanization, while the other undergoes programmed cell death to form the primary appendage (*). Ascospores were observed 4 h after deposition onto germination medium at 27°C. Germination proceeds only on a special medium rich in ammonium acetate through the germ pore (arrow), which is located opposite of the dead cell. The picture shows the germination of a typical wild-type ascospore with its germination peg. (C) Appressoria are formed at the tip of the germ tube issued from the germination of a three-cell spore produced by asexual reproduction on contact with an hydrophobic hard surface (8). The single-cell appressorium strongly adheres to the plant surface and penetrates its host through the formation of a penetration peg at a basal pore as depicted in the picture.

agar (Fig. 4). On the contrary, *P. anserina* ascospores germinate only on specific inducing media at a germ pore (3) (Fig. 4) and germination starts by the extrusion of a spherical isotropic cell, the germination peg, which further differentiates into polarized hyphae (Fig. 2). Our results thus suggest that Pls1 tetraspanins are involved in a cellular process specifically required for the germination of the melanized ascospores from *P. anserina* that is not needed for the germination of the nonmelanized ascospores from *M. grisea*. The *P. anserina* ascospore structure and mode of germination may be dictated by the necessity for these cells, unlike those of *M. grisea*, to be resting and melanized in order to withstand their passage through the digestive tract of herbivores. The relief from this resting stage requires a trigger mimicked by the specific nutritional conditions of the germination medium. One can hypothesize that, in nature, this trigger would be the passage through a digestive tract.

Comparison of *PaPls1* and *PaNox2* mutant defects. In *P. anserina*, the NADPH oxidase 2 (PaNox2) is essential for ascospore germination (36). Like *PaPls1*, *PaNox2* is expressed throughout the life cycle. Nevertheless, the phenotypes of the Δ*PaPls1* and Δ*PaNox2* deletion mutants are similar and restricted to the same early stage of the germination of melanized ascospores. A possibility is that both genes are required during vegetative growth only when *P. anserina* encounters special conditions in nature not present on petri plates. Attempts to assay the superoxide burst in Δ*PaPls1* ascospores, as previously described for the wild type and Δ*PaNox2* mutant (36), were unsuccessful, as this assay proved highly variable (P. Silar, unpublished data). Therefore, it was not possible to test whether PaPls1 is necessary to generate a superoxide burst in ascospores or not. Similarly, the identities of phenotypes exhibited by the two mutants prevented us from performing epistasis analysis. Further experiments are thus needed to know whether PaPls1 and PaNox2 are either involved in the same pathway or in different pathways controlling the same morphogenetic process. Recent studies of orthologues of *PaNox2* in *M. grisea* (*MgNOX2*) and *B. cinerea* (*BcNOX2*) have

shown that these genes are also required for appressorium-mediated penetration (15, 41). The phenotypes of *NOX2* and *PLS1* null mutants are similar in these two fungi (penetration-deficient appressoria), supporting our hypothesis that they are involved in the same specific cellular process. This hypothesis is reinforced by the fact that both *PLS1* and *NOX2* genes have been conserved in the same species during fungal evolution.

Δ*PaPls1* and Δ*PaNox2* ascospore germination defect is suppressed by the inhibition of melanin biosynthesis. The germination of nonmelanized ascospores from the *pks1-193* mutant occurs on both inducing and noninducing media (36). Then, on germination medium, it carries on as in wild-type melanized ascospores. The only observed differences between the *pks1-193* mutant and the wild type are that the emergence of the germination peg happens earlier and that this cellular extrusion reaches a larger size in nonmelanized ascospores. Therefore, the absence of melanin mainly facilitates germination of ascospores, either by triggering the germination process or relieving its inhibition, but does not change the morphogenetic process itself. We have shown that the melanin deficiency of the *P. anserina pks1-193* mutant suppressed the Δ*PaPls1* ascospore germination defect, as observed for Δ*PaNox2* (36). These results further confirm that *PaPls1* and *PaNox2* are involved in a cellular process specifically required for the germination of melanized ascospores. They could be either required for the triggering of ascospore germination on inducing medium or required after this trigger for proper germination. One simple hypothesis compatible with these observations is that PaPls1 and PaNox2 are both involved in the same pathway responsible for the weakening of the germ pore in melanized ascospores, which is not needed in nonmelanized ascospores. For example, this pathway could control the inhibition of the deposition of the melanin layer at the pore or promote the degradation of the pore cell wall. According to this hypothesis, Δ*PaPls1* and Δ*PaNox2* melanized ascospores could have a pore cell wall that cannot be degraded, preventing the extrusion of the germination peg.

The fact that the defect in appressorium-mediated penetration of the *M. grisea MgPLS1* null mutant is not suppressed by the absence of melanin is not too surprising. Indeed during *P. anserina* ascospore germination, a physical barrier does not impair the extrusion of the peg, at least on agar media, allowing the germination peg to be easily extruded from the nonmelanized ascospore. On the contrary, in *M. grisea*, appressoria are firmly attached to the plant surface and the penetration peg has to breach a strong physical barrier to "germinate." This is impossible in nonmelanized appressoria and likely explains the lack of suppression of the *Mgpls1*$^{-}$ mutant defect by melanin inhibition. Melanin-deficient mutants are still able to attack wounded leaves by an unknown mechanism. This was not observed in melanin-deficient *MgPLS1* null mutants that are unable to attack wounded leaves.

Comparison of *P. anserina* ascospore germination and *M. grisea* appressorium-mediated penetration. Since the only defects observed in *P. anserina* Δ*PaPls1* and Δ*PaNox2* mutants as well as *M. grisea* Δ*Mgpls1* and Δ*Mgnox2* mutants are, respectively, the lack of ascospore germination and the absence of formation of the penetration peg in mature appressoria, we tentatively suggest that these two apparently different morphogenetic processes have recruited homologous genes (Fig. 4). Many additional lines of evidence support this hypothesis. First, *P. anserina* ascospores and *M. grisea* appressoria both "germinate" through the differentiation of, respectively, a germination peg and a penetration peg after an induction. Second, *P. anserina* ascospores and *M. grisea* appressoria are heavily melanized cells. In both species, the melanin is deposited between the membrane and the cell wall as a dense continuous layer (3, 14, 24). Third, both structures harbor a pore (i.e., a nonmelanized region with a very thin cell wall). In *M. grisea*, the pore is located at the base of the appressorium in direct contact with the plant surface. In *P. anserina*, the germ pore is located at one tip of the ascospore at the opposite end of the primary appendage (3, 35). In *M. grisea*, melanin acts as a semipermeable membrane retaining osmolytes required for the generation of its high internal turgor pressure (14, 24). This turgor pressure is required for the perforation of plant cuticle and cell wall by the penetration peg. It is not known if *P. anserina* ascospores generate a turgor pressure. However, *P. anserina* nonmelanized ascospores are very fragile and frequently burst upon contact with a needle or an agar plate, suggesting that these cells may have some internal turgor.

Additionally, besides these shared morphological characteristics, *P. anserina* ascospore germination and *M. grisea* appressorium peg formation rely on similar physiological processes such as the catabolism of lipids. *M. grisea* Pth2 peroxisomal carnitine acetyltransferase (5) and Pex6 protein involved in peroxisome assembly (5) are required for the degradation of lipids stored in the appressorium, and the mutants inactivated for these genes cannot build up sufficient turgor pressure to efficiently penetrate the host plant. In *P. anserina*, peroxisome-deficient mutants are also quantitatively impaired in ascospore germination, likely through a defect in lipid catabolism (6). Another striking feature of *P. anserina* ascospore formation is the fact that the ascospore starts as a bicellular structure. Soon, one cell dies and its cytoplasmic content disappears (3). This is reminiscent of the collapse of the spore associated with the formation of *M. grisea* appressorium (49). Additionally, autophagy genes such as *ATG8* (49) and *ATG1* (34) are required for appressorial lipid catabolism, turgor pressure buildup, and appressorium-mediated penetration in *M. grisea*. In *P. anserina*, similar autophagy mutants display a low ascospore germination rate (C. Clavé, personal communication).

C. lindemuthianum appressoria are less studied than those of *M. grisea*. Their ontogeny, morphology, and physiology suggest that they are homologous to *M. grisea* appressoria. *B. cinerea* appressoria derive from the swelling of a germ tube tip and are less melanized. One common determinant among these apparently different appressoria is the differentiation of a penetration peg, whose formation and penetration of host tissues are controlled by *PLS1* and *NOX2*. Melanized ascospores that germinate through a pore and appressoria that differentiate a penetration peg are produced by a large number of fungi, likely through convergent evolution, as they are scattered throughout the fungal kingdom. The presence of *PLS1*/*NOX2* in these fungal species may result from repetitive convergent selection of the same machinery used for the "germination" of apparently very different cells. The ecological factors involved in the selection of such complex structures and their associated "germination" processes may be an advantage for saprobes to resist herbivore digestive tracts or harsh conditions and for patho-

gens to enter rapidly into host plants by breaching the plant cuticle and cell wall. The recurrent but discontinuous use of the same homologous machinery in unrelated species may have facilitated this convergent evolution observed either at the level of spore morphology (presence versus absence of melanin and germination pores) or through lifestyle-associated morphogenetic processes (presence versus absence of melanized appressoria) in Eumycota, as observed for other morphogenetic characters in the fungal tree of life (27).

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