A Mutant Defective in Sexual Development Produces Aseptate Ascogonia†‡

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The formation of fruiting bodies in filamentous ascomycetes is a process of multicellular differentiation controlled by many developmentally regulated genes. The self-fertile ascomycete Sordaria macrospora represents an excellent model for studying cell differentiation during fungal fruiting body development (reviewed in references 9 and 24).

During the life cycle of S. macrospora, a mature haploid ascospore germinates and produces a mycelium composed of multinucleate hyphal compartments. After 2 to 3 days, coiled female reproductive hyphae, called ascogonia, are formed. Each ascogonium develops further into a more or less spherical protoperithecium composed of pseudoparenchymatous tissue surrounding the original ascogonium (24, 47). Ascogenous hyphae emerge from the ascogonium inside the protoperithecium. Within the ascogenous hyphae, the nuclei pair up to form the dikaryotic state, even though S. macrospora is self-fertile and does not require fertilization with an opposite mating type (11, 49). The transition from the spherical protoperithecium to the flask-shaped perithecial stage, is believed to be stimulated by the formation of the dikaryon, although this has not been experimentally verified (24). The dikaryotic state in individual hyphal compartments of the growing ascogenous hyphae is maintained by precisely orchestrated crozier formation from the tip of an ascogenous hypha, fusion of the crozier tip with the penultimate cell of the ascogenous hypha, conjugate mitotic divisions, and highly regulated septation (50, 69). Karyogamy of two nuclei in the penultimate cell results in the formation of a diploid ascus mother cell. The diploid state is very short-lived because the diploid nuclei which are formed immediately undergo meiosis, followed by an extra mitotic division resulting in an ascus containing eight haploid ascospores (11, 49). An identical process has been observed in the heterothallic fungus Neurospora crassa, with the exception that cell fusion between opposite mating types is required prior to dikaryon formation, and each dikaryotic cell compartment within the ascogenous hyphae contains two nuclei of different mating types (43, 50).

Although the cytology of ascogenous hyphae with subsequent crozier formation and karyogamy is well described (3, 43), little is known about the nuclear behavior and septation in the ascogonium or the transition from multinucleate ascogonia to ascogenous hyphae. Recently, we generated a large set of S. macrospora developmental mutants with defects in fruiting body (perithecium) morphogenesis (21). Mutants of the “pro” type were characterized by forming only protoperithecium and therefore have sterile phenotypes. Here, we have characterized the pro22 mutant and show that, in contrast to all other characterized female sterile mutants of Sordaria or Neurospora, it is defective in septum formation in its ascogonia. To our knowledge, this is a novel phenotype for a sterile fungal mutant that will allow the analysis of the role of septation during the earliest stages of sexual morphogenesis in Sordaria and related ascomycetes.

MATERIALS AND METHODS

Strains and culture conditions. The S. macrospora mutant pro22 was generated by ethyl methanesulfonate mutagenesis of the wild-type strain S48977, as described previously (39). All S. macrospora strains used in the present study are listed in Table 1.

Standard growth conditions and DNA-mediated transformation experiments with recombinant plasmids were performed with the mutant strain pro22 as described previously (10, 26, 31, 64). Culture methods used to compare pro22 transcript levels under conditions allowing sexual versus vegetative development and DNA isolation for quantitative real-time PCR analysis have been described by Nowrousian and Cebula (29).

Propagation of recombinant plasmids was performed by using standard labo-
For microscopy with the AxioImager microscope (Zeiss, Jena, Germany), S. macrospora strains were grown on glass slides in glass petri dishes at 27°C with continuous light for 2 to 6 days, as described previously (10). Samples for confocal laser scanning microscopy were prepared by using the inverted-agar-block method (17). Fungal cell walls were stained with 0.1 mg of Calcofluor White/ml (0.1 mg/ml in 0.01% 0.4 M NaOH).

Fluorescence and light microscopic investigations with the AxiosiImager microscope were carried out using an HBO 100 Hg or XBO 75 xenon lamp for fluorescence excitation. For detection of enhanced green fluorescent protein (EGFP) and tdimer2 fluorescence, the Chroma filter sets 41017 (excitation filter HQ470/40, emission filter HQ525/50, and beamsplitter Q560Ip) (Chroma Technology Corp.) were used. For the detection of Calcofluor, the Chroma filter set 31000v2 (excitation filter D350/50, emission filter D460/50, and beamsplitter Q495Ip) (Chroma Technology Corp.) was used. Images were captured with a Photometrix Cool SnapHQ camera (Roper Scientific) and MetaMorph and Adobe Photoshop CS4.

For confocal laser scanning microscopy, the Bio-Rad Radiance 2100 system equipped with a blue diode and argon ion lasers mounted on a Nikon TE2000U Eclipse inverted microscope was used (Bio-Rad Microscience, Hemel Hempstead, United Kingdom). EGFP was imaged by excitation at 488 nm and fluorescence detection at 500/30 nm. A 100×/1.40 NA oil Plan Apochromat objective lens was used for imaging (Nikon). Images were captured with LaserSharp 2000 software v5.1 (Bio-Rad Microscience), and image analysis was performed by using ImageJ.

### TABLE 2. Plasmids used in this study

| Plasmid | Characteristics | Source or reference |
|---------|-----------------|---------------------|
| pANsCos1 | Cosmid vector | 36 |
| D2 | 40.5-kb S. macrospora genomic DNA in pANsCos1 | 41 |
| pDrive | Cloning vector (cloning of PCR fragments) | Qiagen, Hilden, Germany |
| pCR6.1-10 | 4.5-kb pro22 ORF, including the 5′-flanking region in pDrive | This study |
| pEH3 | gpd(p)::egfp | 32, 40 |
| pGFP-22-1 | Tagged with egfp in the NcoI site in pEH3 | This study |
| pRFP-vam-3 | N. crassa vam-3 tagged with tdimer2 in pMF334 | 5 |

### TABLE 3. Oligonucleotides used in this study

| Oligonucleotide | Sequence (5′–3′) |
|----------------|-----------------|
| 3305.................ATCCATGGTGTACTCCACCCTCGAGCTTC | 3306.................ATCCATGGCATCATGAACGCCTC |
| 160.1..................AACCGTCTTCAACTCCACCCTCGAGAC | 165.1..................AACCGTCTTCAACTCCACCCTCGAGCC |
| RT-22-FF.............ACCTTCGCGGCTACCTAA | RT-22-RF.............GTGAGCACCGGCTAGTTT |
| SSU-for..................ATCAAGAGAACAGCACCAGC | SSU-rev..................TGGAGCTGGAATTACCCGC |
RESULTS

The sterile mutant pro22 exhibits a pleiotropic morphogenetic phenotype. Recently, we generated a large set of S. macrospora mutants showing defects in fruiting body maturation (21). We describe here a mutant (pro22) that forms defective protoperithecia that never mature into perithecia. Light microscopy of vegetative hyphae of the wild type and mutant pro22 (Fig. 1) indicated that the mutant was defective in hyphal fusion as previously reported (53). Although adjacent hyphae of the pro22 mutant often made contact in the mature colony, they were never observed to fuse (Fig. 1B) under the experimental conditions used.

Like the wild type, pro22 produced ascogonia after 2 days of growth, which developed further into young protoperithecia (Fig. 2A to D). After reaching a diameter of ~40 μm (Fig. 2C for the wild type and Fig. 2D for mutant pro22), protoperithecia of the mutant strain did not develop any further and thus led to a sterile phenotype. Externally, the protoperithecia of the wild type and pro22 looked very similar. However, after staining their cell walls with Calcofluor White, two-photon laser scanning microscopy revealed that the protoperithecia of the two strains clearly had different internal structures (Fig. 3). Figure 3A depicts a schematic overview of the five optical planes used to scan through each protoperithecium. The fruiting body primordium of pro22 consists of a loose aggregation of thin-walled hyphae without any obvious differentiation of the base of the ascogonium adjacent to the parent hypha, the aggregated hyphae in the wild-type protoperithecia were more densely packed, and many fluoresced stronger with Calcofluor, indicating that they had thicker cell walls (Fig. 3B, upper row).

Although pro22 forms ascogonia like the wild type, on closer inspection we observed a clear difference between the strains. In excess of 200 ascogonial coils of both mutant and wild type were assayed. With the exception of an occasional septum at the base of the ascogonium adjacent to the parent hypha, the ascogonia of pro22 consistently lacked intercalary septa (Fig. 4A), whereas wild-type ascogonia always formed one or more intercalary septa (Fig. 4B). By capturing “z-stacks” of images, we were able to rigorously analyze the three-dimensional structure of the ascogonia, which further confirmed the complete absence of intercalary septa in pro22 ascogonia (see Movies S2 and S3).

**FIG. 1.** (A and B) DIC microscopy of the subperipheral region of the young colony in S. macrospora, 5 to 10 mm in from the colony edge of a 24-h-old culture, inoculated with a mycelial plug. Self-fusion is indicated (circles) and in each case was verified by live-cell observations of cytoplasmic flow through the fusion points. Contacts between hyphae, with no evidence of fusion, are also indicated (by stars). (A) Wild-type strain showing vegetative hyphal fusion (circles), but also a few places of contact (stars), without fusion. (B) In the mutant pro22, there are many points of contact, but no evidence of fusion. Bar, 50 μm. Strains were grown on solid BMM fructification medium.

**FIG. 2.** (A) Ascogonium (ac) of the wild type (wt), which is just starting to be enwrapped by an enveloping hypha (ev). (B) Ascogonium (ac) of the mutant pro22. (C) Protoperithecium of the wild type with a diameter of ~40 μm. At this point, enveloping hyphae have wrapped around the structure. (D) Protoperithecium of the mutant pro22 with a similar diameter of ~40 μm. Further development of pro22 protoperithecia is blocked at this stage. Bar, 10 μm. Strains were grown on solid BMM fructification medium.

Two photon microscopy was performed by using a LSM 510 Meta system mounted on an Axioskop 2 microscope (Zeiss). Calcofluor White was excited with a 780-nm line. Fluorescence emission was detected by using a KP 685 short-pass filter.

**LTSEM.** Samples for low-temperature scanning electron microscopy (LTSEM) were cultured on cellophane (uncoated Rayophane) over agar (52). The cellophane was adhered to the stub with Tissue-Tek (Sakura Finetek, Stanstead, United Kingdom), and specimen preparation was as described previously (51, 52) by using the Hitachi S-4700 cold field emission scanning electron microscope fitted with a Gatan Alto 2500 cryospecimen system. The specimen was partially freeze-dried in the microscope by bringing the temperature of the microscope cold stage up to approximately 50°C and then sputter coated with 60%/40% gold-palladium alloy in the cryopreparation unit.

**Sequence analysis.** Protein sequence alignments were performed by using the CLUSTAL W program (60; http://clustalw.genome.jp). Prediction of transmembrane domains was done by using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TopPred (http://mobyle.pasteur.fr/cgi-bin/portal.py).

**Nucleotide sequence accession number.** Sequence data for the S. macrospora pro22 gene have been submitted to EMBL database under accession number AJ627567.1.
and S3 in the supplemental material). This contrasted with septation in the pro22 vegetative mycelium, which was remarkably similar to that of the wild type (see Fig. S1 in the supplemental material). Thus, the lack of any septa inside the coiled ascogonium is a novel phenotype that is very distinctive for pro22. Interestingly, a homologue of PRO22 from Ashbya gossypii, AgFar11, is directly involved in septum formation during the development of sporangium-like structures (23), indicating that a function in septation during a distinct phase of the life cycle might be conserved.

To determine whether this nonseptate phenotype is a general feature of pro mutants, we investigated ascogonia from four other well-characterized pro mutants: pro1, pro11, pro40, and pro41 (10, 26, 30, 39). As shown in Fig. 4C to F, all of these pro mutants produced septate ascogonia that closely resembled those of the wild type (Fig. 4B). As shown later, this distinctive feature of aseptate ascogonia in pro22 was further confirmed by complementation studies in which the pro22 gene was used to restore the wild-type septate, ascogonial phenotype in the pro22 mutant (Fig. 4G).

Characterization of the complementing pro22 ORF. The pro22 gene was identified by a previously described genomic complementation strategy (26, 39) with an indexed cosmid library representing the S. macrospora genome (41). A 40.5-kb cosmid clone, designated D2, was isolated due to its ability to restore perithecium and ascospore formation in pro22. In subsequent complementation experiments, we succeeded in isolating a 3.3-kb subfragment of D2 that was sufficient to restore fertility in the mutant. Oligonucleotides 165.1 and 160.1 (Table 3) were used for PCR amplification of the entire S. macrospora gene, including the 5’ flanking region (pCR6.1-10, Table 2). We designated the complementing gene as pro22, which is highly homologous to the ham-2 gene from N. crassa, showing 88% sequence identity at the nucleotide level for the coding region. Similar to ham-2, the pro22 ORF comprises 3,613 bp, interrupted by four introns of 58, 65, 60, and 67 bp, which was confirmed by sequencing of the corresponding cDNA fragments.

For a detailed analysis of the mutant locus, genomic DNA from pro22 and the wild type was isolated and subjected to Southern hybridization analysis, using the insert of pCR6.1-10 as a probe. Comparison of the hybridization patterns of genomic DNA digested with EcoRI or HindIII revealed no differences between pro22 and the wild-type strain (data not shown). We therefore concluded that no major rearrangements or deletions occurred in pro22 during chemical mutagenesis.

To investigate whether pro22 does indeed contain a mutation within the pro22 ORF, overlapping genomic DNA fragments from the mutant were amplified by PCR, sequenced and compared to the wild-type pro22 sequence. The mutant pro22 gene differs from that of the wild type by a C-to-T transition within the coding region, changing a glutamine codon to a stop codon. It can therefore be predicted that only a truncated PRO22 polypeptide of 436 amino acid residues is synthesized by pro22 (Q437STOP).

Characterization of the PRO22 protein. The complementing pro22 gene encodes a putative protein of 1120 amino acids with a calculated molecular mass of 124 kDa. BLAST searches (1) in public databases identified several predicted proteins as putative PRO22 orthologs in various eukaryotes ranging from budding yeast to human. The N. crassa homologue of PRO22, HAM-2, is involved in hyphal fusion and sexual development (68). In Saccharomyces cerevisiae, the homologous protein Far11 belongs to the Far complex and plays a role in pheromone-induced cell cycle arrest (20). However, no homologues were detected in genomic sequences from plants and bacteria. The most conserved sequence within the PRO22 homologues is a domain of unknown function found in the C-terminal region of the protein shown in Fig. 5A. A sequence alignment of this domain with homologues of other eukaryotes is depicted in Fig. 5B. By using in silico analysis, we detected three
putative transmembrane domains in PRO22 at amino acid positions 364 to 384, 655 to 673, and 1086 to 1104 (Fig. 5A).

For complementation analyses (Fig. 5A), pro22 was transformed with pGFP-22-1 (Table 2) coding for a PRO22-GFP fusion protein. We obtained two transformants, T548 and T549, which showed restoration of the wild-type phenotype with mature fruiting bodies that released viable ascospores. Microscopy of the ascogonia of these transformants revealed that both developed normal-looking ascogonia with intercalary septa (Fig. 4G). These observations clearly demonstrated that aseptate ascogonia are a specific phenotype of pro22 and that this phenotype is strictly correlated with the pro22 mutation.

Next, we generated p22D153-631 with a 1.5-kb in-frame deletion in the N terminus of pro22 but still encoding part of the PRO22 C terminus. Transformants were still sterile, suggesting that essential domains associated with perithecium development are located within the region deleted in p22D153-631 (Fig. 5A).

Expression analysis of pro22. In common with many other developmental genes, pro22 is only weakly expressed and is not detectable in Northern hybridizations with poly(A)-RNA (data not shown). Using reverse transcription-PCR analysis, we were able to amplify cDNA fragments lacking intron sequences, thus demonstrating transcriptional expression of pro22 (data not shown). Subsequently, we performed a comparative analysis of pro22 transcript levels between wild-type mycelia grown under conditions allowing sexual development versus conditions that only result in vegetative growth. pro22 expression was found to be increased up to 4-fold after 4 days during sexual development (Fig. 6). We further analyzed pro22 expression in mutant pro22, which was more than 4-fold reduced, indicating a positive autoregulation (data not shown).

Subcellular localization of PRO22 in living hyphae. Recently, we have successfully used the EGFP reporter for the subcellular localization of developmental proteins in S. macrospora (10, 30, 40). Attempts to express fluorescent protein-tagged PRO proteins under the control of their native promoters gave very weak signals when imaged by fluorescence microscopy (10). Furthermore, pro22 is weakly expressed transcriptionally (see above) and therefore, as in previous approaches, the Aspergillus nidulans gpd promoter (42) was used to express pro22 fused at its 3′ end with egfp. This resulted in the construction of the plasmid pGFP-22-1 (Table 2), which was used to transform the pro22 strain. We obtained several transformants, with a fertile phenotype, thus indicating that the fusion construct is functional. For confocal laser scanning microscopy, two different transformants, T548 and T549, and four single spore isolates of T548 (T548A to T548D, Table 1) were used. In these strains, fluorescence was restricted to dynamic tubular and vesicular structures in the peripheral region of the colony close to growing hyphal tips although fluorescence was absent from a 30 to 40 μm zone immediately behind these tips (Fig. 7B and D). This tubular network with associated vesicular structures is similar in morphology and dynamics to the vacuolar system typically found in the peripheral region of filamentous fungal colonies (8, 63). However, other organelles (e.g., mitochondria and endoplasmic reticulum [ER]) can exhibit a tubular and/or net-like organization in apical hyphal compartments but, significantly, these organelles are not excluded from the extreme apical regions of hyphae (12, 16, 17, 25). Localization experiments with mitochondrial and ER markers confirmed that PRO22 is neither localized to the ER nor mitochondria (data not shown).

To verify localization of PRO22 to vacuoles in S. macrospora, we used VAM-3, a vacuolar membrane protein, which...
FIG. 5. (A) Physical map of PRO22 (top) encoded by the pro22 ORF (bottom). Three putative transmembrane domains are marked in light gray, and a conserved domain of unknown function is shown in dark gray. The pro22 ORF is indicated by a gray arrow; the introns are highlighted in white. Recombinant plasmids are shown below the physical map and were used for complementation analysis with the mutant pro22 as recipient. Numbers show the positions of amino acids (aa) and nucleotides (bp). Fertility is indicated by "+/H11001", while "−" marks sterility. (B) Alignment of the conserved C-terminal domain, positions 714 to 1008 of the S. macrospora peptide, containing highly conserved residues. The first two characters on the left indicate the name of an organism: Sm (S. macrospora, accession no. AJ627567 [NCBI]), Nc (N. crassa, accession no. CAC28842 [NCBI]), Mg (Magnaporthe grisea, accession no. MG007311 [Broad Institute]), An (A. nidulans, accession no. XM_659123 [NCBI]), Fg (Fusarium graminearum, accession no. XP_387335 [NCBI]), Um (Ustilago maydis, accession no. XP_758764 [NCBI]), Ce (Caenorhabditis elegans, accession no. AAA82421 [NCBI]), Dm (Drosophila melanogaster, accession no. NP_467806 [NCBI]), Hs (Homo sapiens, accession no. AAH19084 [NCBI]), Sc (Saccharomyces cerevisiae, accession no. NP_014272 [NCBI]). Amino acids that are invariant are highlighted in black. Identical residues conserved in more than 50% of the aligned sequence are highlighted in dark gray, while conserved similar residues are highlighted in light gray. Sequence analysis was performed as described in Materials and Methods.
has already been used as a vacuolar marker in different filamentous fungi (5, 57). Since *N. crassa* and *S. macrospora* VAM-3 show a high level of sequence similarity (34, 35), we were able to use the *N. crassa* VAM-3 (5) for colocalization experiments with PRO22. In order to generate strains for colocalization, we performed cotransformation of mutant pro22 with pGFP-22-1 and pRFP-vam-3 (Table 2). The latter contained a 3’ terminal fusion of the *N. crassa* vam-3 with tdimer (5). Transformants obtained in this experiment regained fertility and, for microscopic investigations, transformant T556, expressing both PRO22-GFP and the *N. crassa* RFP-VAM-3, was chosen. Both fusion proteins colocalized in the same tubular and vesicular structures close to hyphal tips (Fig. 8), confirming that PRO22 is localized in the vacuolar system of the peripheral colony region. As described previously (5),

FIG. 6. Quantitative real-time PCR analysis of *pro22* transcript levels. The wild type was grown under vegetative and sexual growth conditions. The data are shown as base 2 logarithmic ratios for the mean values (calculated using REST) of three independent experiments.

FIG. 7. PRO22 localization in living vegetative hyphae of *S. macrospora*. (A) Scheme of vacuolar distribution in hyphal compartments. Dynamic tubular and vesicular vacuoles are restricted to the peripheral region and are absent from the very tip. These vacuoles disappear in the subperipheral region and are replaced by more static large spherical vacuoles often stuck at septal pores. (B) Fluorescence microscopy of a growing hyphal tip showing that the PRO22-GFP only starts to label tubular and vesicular components of the vacuolar system 30 to 40 μm back from the hyphal tip. (C and D) Fluorescence microscopy showing localization of PRO22-GFP in dynamic tubular and vesicular components of the vacuolar system in the peripheral region of the colony (D) but its absence from the large spherical vacuoles in the subperipheral colony region (C). Bar, 10 μm. Strains were grown on solid BMM fructification medium.

FIG. 8. DIC and fluorescence microscopy showing the localization of PRO22-GFP and the *N. crassa* vacuolar marker RFP-VAM-3 in tubular and vesicular components of the vacuolar system in vegetative hyphae from peripheral regions of the colony (A) and in ascogonia (B). The merged images shows colocalization of the fusion proteins confirming that PRO22-GFP is a vacuolar protein. Bar, 10 μm. Strains were grown on solid BMM fructification medium.
RFP-VAM-3 not only localizes to the vacuolar membrane but also to the vacuolar lumen, as was observed for PRO22-GFP.

Because of our observed involvement of PRO22 in septum formation in ascogonia and the pro22 mutant being defective in sexual development, we analyzed these sexual structures of S. macrospora to determine the localization of PRO22-GFP in them. The DIC micrographs clearly demonstrated the existence of vacuoles in ascogonia (Fig. 4). Fluorescence of PRO22-GFP was observed in large and small spherical structures, strongly resembling vacuoles (Fig. 8B). Furthermore, RFP-VAM-3 clearly colocalized within these structures, indicating that the stained structures are indeed vacuoles (Fig. 8B). In summary, PRO22 is not only localized to the dynamic tubular and spherical vacuoles at the periphery of the colony but also in ascogonia in the subperipheral region.

Besides tubular and vesicular vacuoles, filamentous fungi also possess large spherical vacuoles that are mostly static and commonly positioned adjacent to, and on the distal side of, septa (5, 8, 56). PRO22-GFP was found to be absent from these spherical vacuolar compartments (Fig. 7C). Figure 7A summarizes the structure and distribution of vacuoles in diagrammatic form as it is for S. macrospora and other filamentous fungi. In contrast to PRO22-GFP, N. crassa VAM-3 is localized in both the large spherical vacuoles and the tubular/vesicular vacuoles (data not shown).

**DISCUSSION**

**Septum formation in ascogonia is a prerequisite for fruiting body formation.** In the present study, we used a forward genetic approach (21, 26, 39) to characterize the sterile S. macrospora mutant pro22, which forms protoperithecia but not mature perithecia. Scanning electron microscopy and extensive live-cell imaging confirmed our previous observation that pro22 is defective in hyphal fusion (53) and that this defect is correlated with sexual development. A link between defects in sexual development and hyphal fusion has been previously reported in mutants of S. macrospora and N. crassa (10, 50, 53), suggesting a functional relationship between hyphal anastomosis and fertile fruiting body formation. In the wild type, multiple hyphal fusion events result in an interconnected hyphal network that permits translocation of cellular components, such as water, organelles, metabolites, nutrients, or signaling molecules throughout the colony, which presumably facilitates growth and reproduction (6, 13, 46, 50). Transport of cellular components may be one function of hyphal fusion, but hyphal fusion may play other roles during sexual development. It may, for example, be necessary for the formation of compact, highly differentiated, interconnected, multicellular fruiting bodies. The lack of hyphal fusion may be one of the reasons why pro22 forms loosely aggregated protoperithecia, and its reduced interconnected state may play a role in causing early developmental arrest.

Our observation that vegetative hyphae of pro22 are septate while ascogonia lack septa seems contradictory. However, this indicates that PRO22 may play a specific role in septation during sexual development. Each septum in vegetative hyphae contains a septal pore that can allow cytoplasmic flow between adjacent hyphal compartments and distribution of organelles throughout the whole colony. Occlusion of septa during sexual development may serve other important functions. In ascogenous hyphae of Sordaria humana it has been shown that the septal pores have highly complex and sometimes very elaborate membranous structures associated with them. These pore occlusions are of a type not found in vegetative hyphae and are highly variable structures from being relatively simple pore caps to having complex swollen rims with associated membrane eisternae (2, 49). These membranous structures provide continuity between the endomembrane systems of adjacent cells in asogenous hyphae and particularly between the nuclear envelopes of the two nuclei in the same cell and between nuclei in adjacent cells. This suggests that they play a specialized role in communication in asogenous hyphae that may, in some way, relate to the regulation of the dikaryotic state (2, 49). An important aspect of the plugging of septal pores is that they may also physically isolate adjacent hyphal compartments and thus allow them to undergo different modes of differentiation (15). In this way septation and septal pore plugging must play important roles in the differentiation of fungal multicellular structures such as perithecia (48). However, at present we do not know whether the septa of ascogonia possess septal pores, and if they do, whether they are plugged and by what type of septal pore occlusions. We speculate that plugged septa in ascogonia might allow for the accumulation of signaling molecules which trigger subsequent sexual development. Aseptate ascogonia in pro22 may therefore prevent such an accumulation, and this could explain the sterile phenotype.

The homologue of PRO22 in S. cerevisiae, Far11, plays a role in pheromone-induced cell cycle arrest during the life cycle of yeast and forms a complex with other Far proteins regulating G1 arrest (20). Kemp and Sprague (20) further suggested that Far11 and the other members of the Far complex may be required for cell fusion during mating. They have proposed that the Far complex could serve as a checkpoint that monitors fusion and prevents premature re-entry into mitosis to ensure that mating cells have sufficient time to fuse. Whether or not PRO22, like its yeast homologue, is directly involved in the regulation of mitosis remains to be determined.

It is very interesting that in S. cerevisiae, the PRO22 homologue Far11 directly interacts with the small GTPase rho4 (62). Rho4 is involved in septation in many ascomycetes; it was first observed in the fission yeast Schizosaccharomyces pombe, where Δrho4 mutants have multiple, abnormal septa (28, 55). In contrast, a rho4 mutation in N. crassa leads to a complete loss of septation in vegetative hyphae (44). Rasmussen and Glass further showed that RHO-4 localizes to septa and identified a difference in the regulation of septation during conidiation versus vegetative septation in N. crassa (44, 45). Recent studies in N. crassa and A. nidulans indicate that RHO-4 localizes to septa and to the plasma membrane and is involved in signaling pathways that regulate septum formation (19, 58).

Another homologue of PRO22 is AgFar11 from A. gossypii, which is directly involved in septation formation during the development of sporangium-like structures. Normally, in A. gossypii, formation of these “sporangia” involves hyphal abscission by a constriction at the septum between the old mycelium and the sporangium (67). Interestingly, deletion of the Agfar11 gene led to a defect during septation formation with complete abscission of hyphal compartments (23). Thus, in ΔAgfar11,
this process already occurs before sporangium formation. This phenotype indicates a role for AgFar11 in the regulation of septum formation that is dependent on the developmental stage of the fungus. Interestingly, the pro22 mutant lacks septa in ascogonia but forms them in vegetative hyphae. Therefore, the loss of septation in ascogonia suggests that PRO22 in S. macrospora has a different function during the sexual phase of the life cycle compared to its role in the vegetative mycelium.

PRO22 is associated with the dynamic vacuolar network near growing hyphal tips and with vacuoles in ascogonia. In our attempt to localize PRO22, we analyzed different S. macrospora transformants expressing a functional EGFP-tagged version of the protein. Our finding that PRO22 does not localize to the entire vacuolar system of S. macrospora, but specifically to the dynamic tubular and vesicular vacuolar compartments near growing hyphal tips in the peripheral region of the colony and to vacuoles in ascogonia strongly suggests that the protein has a unique function in these organelles. It has been previously proposed that the different types of vacuoles may play different roles in different regions of the mycelium (56, 57).

For colocalization experiments, we used the well-established vacuolar membrane protein VAM-3 from N. crassa (5). Since VAM-3 localizes exclusively to the vacuolar membrane in budding yeast (65, 66), it has been used as a vacuolar marker in plants and fungi (5, 22, 57, 61). In N. crassa, VAM-3 localizes to the entire vacuular system, including tubular and vesicular vacuolar compartments (5). The N. crassa VAM-3 not only localizes to the membrane of spherical vacuoles but also to the lumen, where most probably an internalization of the vacuolar membrane components occurs. Bowman et al. (5) proposed that this might be due to a protein turnover process.

A VAM-3 homologue was also used as a vacuolar marker in A. oryzae by labeling AoVam3 with GFP (57). Similar to N. crassa, fluorescence was observed in the entire vacuolar system, including the lumen of large spherical vacuoles in the subperiphery of the colony, although the GFP tag was located on the cytosolic side of the vacuolar membrane. These authors postulated that an autophagic degradation of cytosolic materials might have occurred, followed by a subsequent internalization of the vacuolar membrane into the lumen. Tubular vacuoles might then transport the degraded material to more active regions of the colony.

Significantly, PRO22 could not be identified in the spherical vacuoles of vegetative hyphae in the sub peripheral region of the colony and was only found in vacuoles in ascogonia and in very dynamic components of the tubular and vesicular vacuolar network in the colony periphery. Here, PRO22 was not restricted to the vacuolar membrane but also occurred in the lumen. It is possible that PRO22 plays a role in nutrient transport which has been previously shown to be carried out by the tubular vacuolar system (13), whereas the protein is probably not involved in vacuolar processes occurring in the spherical vacuoles in the more distant regions of the colony. Our findings that PRO22 is localized to vacuoles in ascogonia seems reasonable because the phenotypic characterization of the mutant points to a role in this part of the S. macrospora life cycle.

A recent finding supports our observation that PRO22 is localized to vacuoles. Simonin et al. (59) showed that the N. crassa ham-2 mutant, which corresponds to the pro22 mutant, exhibits an aberrant vacuolar morphology. In ham-2 and other ham mutants deficient in hyphal fusion, large vacuoles were observed in conidial germings, suggesting a defect in vacuolar protein sorting. Interestingly, the S. cerevisiae mutant far11 and two other far mutants, far9 and far10, were identified in a large-scale screen for vacuolar protein sorting mutants (4), indicating that Far11, like its homologues PRO22 and HAM-2, may play a role during this process.

Is there a link between septation and vacuolar localization? Our finding that PRO22 localizes to vacuoles and plays a role during septum formation in sexual structures raises the question as to how its localization and function are linked. A candidate which links septum formation and vacuolar localization might be bnl1 from the fission yeast S. pombe, the homologue of the human batten disease gene CLN3. Bnl1 deletion strains displayed enlarged and more alkaline vacuoles, indicating a role for Bnl1 in the vacuolar system, and this corresponded with the localization of the protein in vacuoles and prevacuolar compartments (14). Furthermore, bnl1 deletion cells had a significantly higher septation index and an increased number of binucleate cells, suggesting a perturbation of cell cycle progression. Although septation in fission yeast and filamentous fungi clearly serves different functions, the molecular mechanisms may have similarities.

In another study, the entire set of S. pombe ORFs was cloned in a global approach to analyze protein localization in fission yeast (27). Interestingly, the PRO22 homologue SpFar11 was shown to localize to vacuoles. This large-scale experiment requires verification by a more detailed examination of SpFar11. However, a connection between this protein and the vacuolar system seems possible and would corroborate our findings.

Taken together, our data point to a specific role for PRO22 in the vacuolar network and an involvement in septation during the initiation of the sexual life cycle. These findings may provide new insights into these very early sexual processes, and future work will be focused on studies to investigate the interaction of PRO proteins controlling multicellular development.

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