

The septal pore cap is an organelle that functions in vegetative growth and mushroom formation of the wood-rot fungus *Schizophyllum commune*

Arend F. van Peer,¹ Fengfeng Wang,¹
Kenneth G. A. van Driel,^{2,3} Jan F. de Jong,¹
Elly G. van Donselaar,² Wally H. Müller,²
Teun Boekhout,³ Luis G. Lugones¹ and
Han A. B. Wösten^{1*}

¹Microbiology and Kluyver Centre for Genomics of Industrial Fermentations, Institute of Biomembranes, Utrecht University, 3584 CH Utrecht, the Netherlands.

²Cellular Architecture and Dynamics, Institute of Biomembranes, Utrecht University, 3584 CH Utrecht, the Netherlands.

³Centraalbureau voor Schimmelcultures (CBS-KNAW), Royal Netherlands Academy of Arts and Sciences (KNAW), Uppsalalaan 8, 3584 CT Utrecht, the Netherlands.

Summary

Mushroom-forming basidiomycetes colonize large areas in nature. Their hyphae are compartmentalized by perforated septa, which are usually covered by a septal pore cap (SPC). Here, we describe, for the first time, the composition and function of SPCs using the model system *Schizophyllum commune*. The SPC of *S. commune* was shown to consist of a proteinaceous matrix covered by a lipid membrane. The matrix was demonstrated to define the ultrastructure of the SPC and to consist of two main proteins, Spc14 and Spc33. Gene *spc14* encodes a protein of 86 amino acids, which lacks known domain, signal or localization sequences. Gene *spc33* encodes a 239 and a 340 amino acid variant. Both forms contain a predicted signal anchor that targets them to the ER. Immunolocalization showed the presence of Spc33 in the SPC but not in ER. From this and previous reports it is concluded that the SPC is derived from this organelle. Inactivation of *spc33* resulted in loss of SPCs and the inability to close septa. The latter may well explain why vegetative growth and mushroom formation were

severely reduced in strains in which *spc33* was inactivated.

Introduction

Filamentous fungi colonize substrates by hyphae that grow at their apices and branch subapically. This mode of growth, together with hyphal fusion, results in an interconnected network of hyphae that is called a mycelium. Hyphae of the lower fungi, i.e. the Glomeromycota, Zygomycota and Chytridiomycota (James *et al.*, 2006), are occasionally, if at all, divided by septa (Barr, 2001; Benny *et al.*, 2001). In contrast, hyphae of the higher fungi, i.e. the Ascomycota and Basidiomycota (James *et al.*, 2006), are septated. Yet, the central pore in these septa allows streaming of cytoplasm and translocation of organelles between hyphae and their compartments (Buller, 1933; Bracker and Butler, 1964). Septa of *Schizophyllum commune* close upon mechanical damage. Moreover, they plug as a response to environmental conditions such as presence of high salt or an antibiotic, or as a response to high temperature (van Peer *et al.*, 2009a). Septa of filamentous fungi also close during sporulation (Berteaux-Lecellier *et al.*, 1998) and heterokaryon incompatibility (Glass and Kaneko, 2003).

The septa of *Basidiomycota* that belong to the *Agaricomycotina* have a barrel-shaped swelling around the pore, which is known as the dolipore. The dolipore of these fungi (among which many mushroom-forming fungi) is generally associated with a septal pore cap (SPC) (Bracker and Butler, 1963). This cap can be of the vesiculate type, the imperforate type or the perforate type (McLaughlin *et al.*, 1995; van Driel *et al.*, 2009). The SPC of *S. commune* belongs to the latter type. Its perforations are approximately 100 nm in diameter and are regularly distributed over the structure (Müller *et al.*, 1994; 1998; 1999). The SPC, which has a diameter of 450–600 nm, consists of a matrix that is surrounded by a membrane (Girbardt, 1958; 1961; Moore and McAlear, 1962; Bracker and Butler, 1963; Marchant and Wessels, 1973; Müller *et al.*, 1998; 2000). At its base the SPC is connected to the endoplasmic reticulum (ER) (Girbardt, 1961; Moore and Patton, 1975; Müller *et al.*, 1998). This and the fact that ER markers (van Driel *et al.*, 2008) and zinc-iodine

Received 3 September, 2009; accepted 26 October, 2009. *For correspondence. E-mail h.a.b.wosten@uu.nl; Tel. (+31) 30 2533448; Fax (+31) 30 2513655.

osmium tetroxide (Müller *et al.*, 1998; 1999; 2000) stain the SPC region suggest that the SPC is derived from this organelle.

Although the ultrastructure of SPCs is well studied, their composition, dynamics and function are largely unknown. Recently, we have shown that the SPC18 protein of *Rhizoctonia solani* not only resides in the SPC but also can be found in the plug that closes the septum upon damage (van Driel *et al.*, 2008). This suggests that the SPC is involved in the plugging process. Here, we describe the purification, and composition of the SPCs of the model basidiomycete *S. commune* and show that it functions in closure of septa and in growth and development.

Results

Purification of the SPCs of *S. commune*

Homogenized mycelium of the $\Delta sc3\Delta sc15$ strain of *S. commune* (for details about the choice of this strain see *Experimental procedures*) was loaded onto discontinuous sucrose gradients and centrifuged at 100 000 *g*. Transmission electron microscopy (TEM) revealed that the SPCs of *S. commune* migrated through 45% (w/w) sucrose but accumulated on top of a 50% solution of the sugar. Cell wall fragments migrated through this concentration of sucrose and pelleted at the bottom of the centrifuge tube. However, vesicles and membrane remnants behaved like the SPCs in density flotation (Fig. 1A). To remove the contaminating lipids, the homogenate was

treated with 1% SDS, 1% Tween-20 or 1% Triton X-100 prior to centrifugation. When treated with SDS, the SPC ultrastructure was disrupted and could no longer be detected by TEM (not shown). SPC ultrastructure was maintained in the presence of Tween-20 (not shown) or Triton X-100 (Fig. 1B). These detergents dissolved most of the vesicles, which thus no longer contaminated the SPC-enriched fraction. This was especially the case in the Triton X-100 treated samples.

Interestingly, the buoyancy of the SPCs increased upon treatment with Triton X-100. The SPCs accumulated at 55% w/w (1.2575 g cm⁻³) sucrose as opposed to 50% w/w (1.2295 g cm⁻³) sucrose before treatment with the detergent. Transmission electron microscopy analysis revealed that the perforations of the SPC changed from round to hexagonal upon treatment with Triton X-100 (inserts in Fig. 1A and B). These data indicate that membranous material is removed from the outer surface of the SPC and that the remaining matrix consists of proteins. This matrix defines the ultrastructure of the SPC.

To further purify the SPCs of *S. commune*, two discontinuous sucrose gradients were combined with a filtration step. Cell wall fragments of high buoyancy (> 1.2575 g cm⁻³, i.e. 55% w/w sucrose) and low-density material (< 1.1764 g cm⁻³, i.e. 40% w/w sucrose) were separated from the SPCs in the first fractionation over a 40–55% discontinuous sucrose gradient. The protein profile of the 40–55% sucrose interface that contained the SPCs was complex (Data not shown). This interface fraction was diluted with 1% Triton X-100 to a final concentration of less than 10% w/w sucrose and loaded on top of

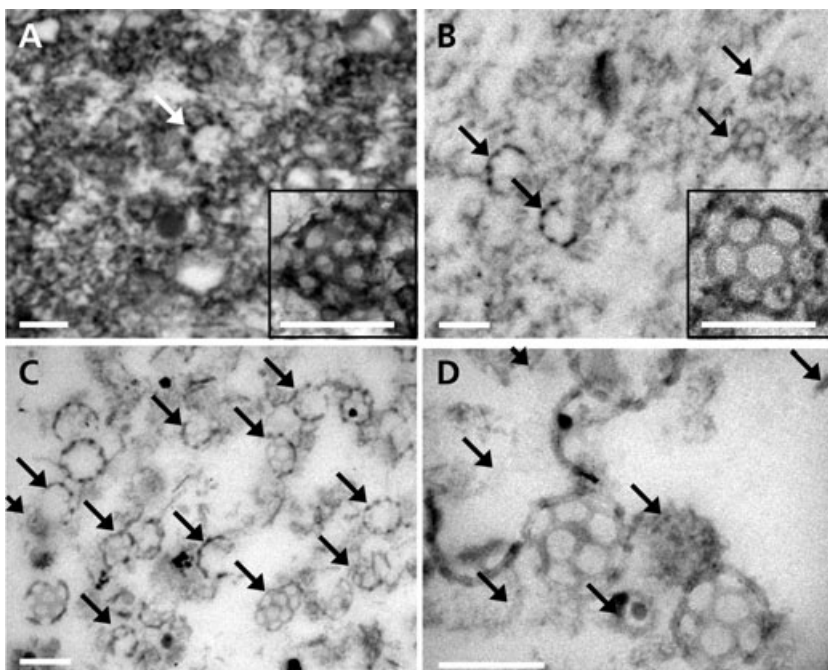


Fig. 1. Transmission electron microscopy of fractions obtained during purification of the SPCs of *S. commune*. Fifty per cent (A) and 55% (B) interface of a sucrose gradient loaded with a mycelial homogenate that was (B) or was not (A) treated with Triton X-100. An SPC in (A) (arrow) is surrounded by vesicles and membranous material. Inserts in (A) and (B) show SPCs at higher magnification with round and hexagonal perforations respectively. (C and D) Purified SPCs after two density gradients and after filtration over a 220 nm filter. Bars represent 1 μ m in A–C and 500 nm in D and in the inserts of A and B.

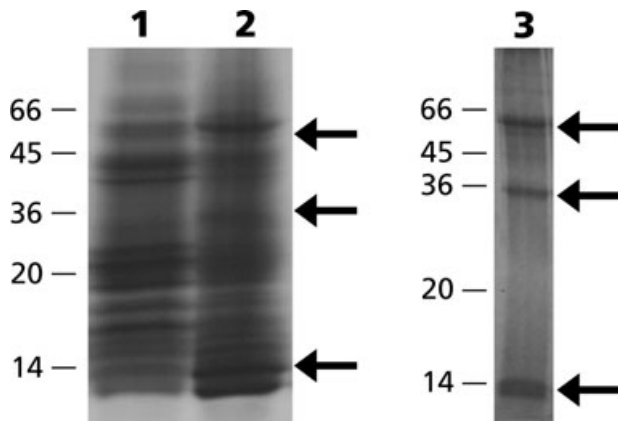


Fig. 2. SDS-PAGE analysis of fractions obtained during purification of SPCs of *S. commune*. 47–52% (lane 1) and 52–70% (lane 2) interfaces of a discontinuous sucrose gradient, and the residue after separation on a 220 nm filter (lane 3). Arrows indicate the positions of the 14, 33 and 60 kDa bands.

a second, three layered, 47%–52%–70% w/w sucrose gradient. The SPCs accumulated on top of the 70% sucrose layer due to their increased density after the Triton X-100 treatment. The protein pattern of the 52–70% interface was clearly different from that of the 47–52% interface but was still complex (Fig. 2, lanes 1 and 2). Therefore, the 52–70% interface fraction was diluted to a final sucrose concentration of less than 10% and passed through a 220 nm cut-off filter. This resulted in a dense layer of SPCs on top of the filter. The SPC containing layer could be removed from the filter by forcing down distilled water through the inverted filter. SPCs were collected as a pellet from this fraction by centrifugation at 10 000 *g*. TEM analysis showed that SPCs were highly enriched in this fraction (Fig. 1C and D). SDS-PAGE of this fraction revealed three abundant protein bands with an apparent molecular weight of 14, 33 and 60 kDa (Fig. 2, lane 3).

Genes spc14 and spc33 encode the main proteins of the SPC matrix

The first two amino acids of the 14 kDa protein could not be identified by N-terminal sequencing. However, amino acids 3–15 revealed the sequence E/DEEILLGIIVSVNHG. These amino acids matched the predicted gene *e_gw1.1.806.1* of *S. commune* (protein ID: 47189; JGI genome database, <http://genome.jgi-psf.org/Schco1/Schco1.home.html>). Analysis of the genomic sequence and a 606 bp cDNA of *S. commune* revealed an open reading frame encoding a 86-amino-acid protein (Fig. 3A). The protein lacks known domain, signal or localization sequences. BLAST search of *spc14* identified homologues in *Phanerochaete chrysosporium*, *Postia placenta*, *Coprinopsis cinereus* and *Laccaria bicolor* (Fig. 3A) as well

as in *Amanita bisporigera*, *Paxillus involutus* and *Pisolithus tinctorius*. These basidiomycetes all belong to the *Agaricomycotina* and are known to possess a perforate SPC. Genes homologous to *spc14* genes were not found outside the *Agaricomycotina*. In fact, even fungi within the *Agaricomycotina* that do not have an SPC (e.g. *Cryptococcus neoformans*) do not contain an *spc14* homologue. Massive parallel signature sequencing (MPSS) showed a distribution of 215 transcripts per million (tpm) in 4-day-old monokaryotic mycelium, 580 tpm in 4-day-old dikaryotic mycelium and 312 tpm in mature mushrooms. This indicates that *spc14* is constitutively expressed.

The N-terminal sequences from the 33 kDa (MHPST-SHH) and 60 kDa (MGPST) protein bands from purified SPCs were quite similar. However, the sequences were too short to identify the encoding genes in the genomic sequence of *S. commune*. Therefore, MS-MS fingerprinting was performed after tryptic in-gel digestion. Both bands displayed a highly similar mass fingerprint (data not shown), indicating that these bands contain the same protein that was called Spc33. Three major peaks that were common in both mass-fingerprints were selected for LC-MS-MS analysis. The peptide sequences (Table 1) did not show a perfect match with any of the open reading frames in the genome of *S. commune*. They did partially match the predicted gene *estExt_Genewise1.C_20499* (protein ID: 74104; JGI genome database; <http://genome.jgi-psf.org/Schco1/Schco1.home.html>). This ORF also contained the N-terminal sequence that was obtained for the 33 and 60 kDa protein bands. Moreover, antibodies raised against peptides encoded by the ORF reacted specifically with the 33 and 60 kDa bands (see below). These data show that we identified the gene encoding these protein bands. The mismatches with the determined peptide sequences may have been caused by contaminating peptides.

By aligning expressed sequence tags (ESTs) contained in the JGI database (<http://genome.jgi-psf.org/Schco1/Schco1.home.html>) two transcripts of *spc33* were found, which encode a 239- and a 340-amino-acid variant (Fig. 3B). MPSS analysis showed that both mRNAs are formed throughout the life cycle of *S. commune*; 14/18//1 and 35/24/41 transcripts per million were detected, respectively, in 4-day-old monokaryotic mycelium, 4-day-old dikaryotic mycelium and mature mushrooms. The first 228 amino acids of the two Spc33 variants are identical (Fig. 3B). These parts contain a predicted signal anchor (aa 1–51) (SignalP 3.0, Nielsen *et al.*, 1997; Bendtsen *et al.*, 2004) and an additional transmembrane domain (aa 106–128) (Mobylye, TopPred program, von Heijne, 1992; Claros and von Heijne, 1994). Phosphorylation sites are also predicted (NetPhos Server 2.0, Blom *et al.*, 1999). Taken together, these data suggest a localization of both Spc33 variants in the ER. The alternative splicing



Fig. 3. Deduced amino acid sequences of Spc14 (A) and both variants of Spc33 (B) of *S. commune* (Sc), *P. chrysosporium* (Pc), *P. placenta* (Pp), *C. cinereus* (Cc) and *L. bicolor* (Lb). Sequences are highly conserved as indicated by shaded areas ($\geq 80\%$ identity). The first 228 amino acids of both variants of Spc33 are identical. Due to alternative splicing of the *spc33* mRNA the C-termini of the Spc33 variants differ. The predicted signal anchor (aa 1–51) and an additional transmembrane domain (aa 106–128) are indicated by thin and thick lined boxes respectively.

of the second intron within the coding sequence results in different C-terminal sequences. The mRNA of the short variant includes this intron and is terminated within this sequence after 11 amino acids. The mRNA of the long

Table 1. LC-MS-MS peptide sequences obtained from mass-peaks identified in both the 33 and 60 kDa protein bands and the corresponding sequences deduced from *spc33*.

	LC MS MS peptide sequences	Spc33 sequence
Peak 1	L/I D L/I TEQ L/I L/I	SWITEQLR
Peak 2	TPPXXVSP	TPPGWHSP
Peak 3	EY L/I HEYFYEE	VEEEYFYEP

variant lacks the intron and terminates in the 113th codon of the third exon. The peptides from the 33 and 60 kDa protein bands that were analysed by LC MS MS were from the part of Spc33 that is shared by both variants. Therefore, it is not clear which protein variant(s) is produced.

BLAST search identified *spc33* homologues only in fungi that belong to the *Agaricomycotina* and that possess an SPC (*C. cinereus*, *P. chrysosporium*, *L. bicolor*, *P. placenta* and *Armillaria* sp.). In all cases, alternative splicing may result in a large and a small variant of the Spc33 protein (Fig. 3B). The overall amino acid identity is $\geq 65\%$.

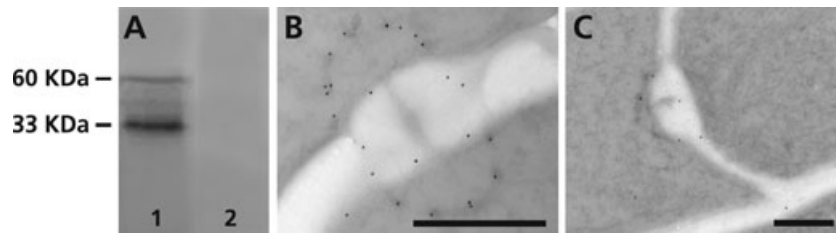


Fig. 4. Localization of Spc33 in hyphae of *S. commune*. (A) Western analysis of SDS-soluble proteins of the wild-type (lane 1) and the Δ *spc33* mutant (lane 2) strain shows that the Spc33 antiserum reacts specifically with the 33 and 60 kDa forms of the septal pore cap protein. This antiserum (B) but not a pre-immune serum (C) reacted with the SPCs of *S. commune* hyphae. Bars represent 500 nm.

Immuno-localization of Spc33

Anti-sera against recombinant Spc14 were not specific and therefore did not allow us to perform an immuno-localization of the protein. In contrast, a specific antiserum against Spc33 was obtained by injecting a mixture of the synthetic peptides EVVNRREHDASRRE and LKVAERERDISKRE that are contained within the 228 amino acids that are shared by the variants of the protein. Indeed, the antiserum reacted with both the 33 and 60 kDa protein bands in *S. commune* wt lysates (Fig. 4A). Immuno-gold labelling showed the presence of Spc33 within the SPC (Fig. 4B and C). Notably, no signal was observed in the ER, not even close to the SPC base.

spc33 deletion strains do not plug and are affected in growth and development

Schizophyllum commune 4–8 was transformed with deletion construct pAVPKO33b. Approximately 500

nourseotricin resistant colonies were obtained, of which about 30% were phleomycin sensitive. The latter colonies were candidates to carry an inactivated *spc33* gene. Indeed, two transformants had a gene deletion based on a PCR showing the absence of an intact *spc33* gene and the presence of an insertion of the nourseotricin resistance cassette in the *spc33* locus (Fig. S1). The gene deletion was confirmed by Southern analysis (data not shown). The mutant strains were crossed with the compatible strain 4–8b. Nourseotricin resistance and absence of the *spc33* phenotype segregated 1:1 in 100 independent siblings, showing that the deletion construct had integrated only once and that mutant phenotypes (see below) are solely due to the inactivation of the *spc33* gene. Electron microscopy revealed that the mutant strains lack a visible SPC (Fig. 5).

Mono- and dikaryons of the wild-type and the Δ *spc33* strain were grown on minimal medium plates. The monokaryotic Δ *spc33* strain grew much slower than the

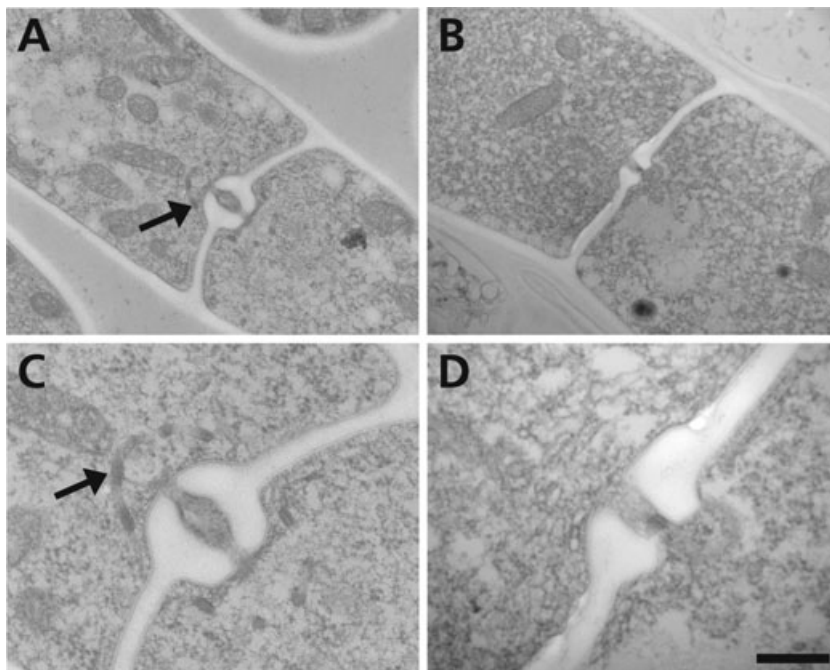


Fig. 5. SPCs (indicated by an arrow) can be observed as a dome-shaped structure around the dolipore of a wild-type strain of *S. commune* (A and C) but not around septa of a strain in which *spc33* has been inactivated (B and D). Bar represents 500 (A and B) and 250 nm (C and D) respectively.

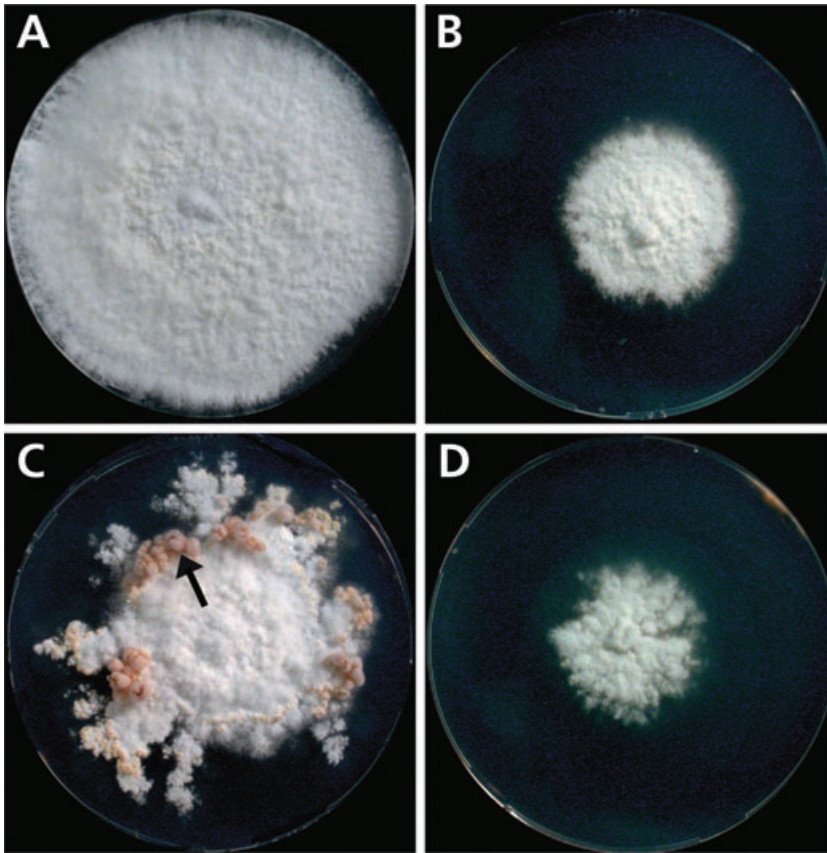


Fig. 6. Morphology of 8-day-old colonies of a wild-type *S. commune* monokaryon (A) and dikaryon (C) and their derivatives in which *spc33* has been inactivated (B and D respectively). The wild-type dikaryon, but not that of the mutant strain, forms fruiting bodies (indicated by an arrow) at 25 or 30°C.

wild-type (Fig. 6). Biomass was reduced with $82 \pm 2\%$. A similar result was obtained with the $\Delta spc33\Delta spc33$ dikaryon (data not shown). In contrast to the wild-type dikaryon, the $\Delta spc33\Delta spc33$ dikaryon did not form fruiting bodies at 25°C or 30°C after 8 days of growth (Fig. 6). This was not caused by the reduced colony size because fruiting bodies were also not formed by 16-day-old colonies of the mutant strain that had a colony size similar to that of an 8-day-old wild-type colony forming reproductive structures (data not shown). The mutant did form fruiting bodies at 18°C. However, the number of fruiting bodies produced by the mutant was much less compared with the wild-type and they were smaller and did not sporulate. Moreover, mutant fruiting bodies were formed over the whole colony surface, whereas the wild-type strain and the heterozygous $\Delta spc33$ dikaryon formed the fruiting bodies in a ring at the edge of the colony.

To assess whether the SPC is involved in plugging of the septal pore, hyphae were grown in a thin layer of medium (for details see *Experimental procedures*; van Peer *et al.*, 2009a). Recently, we have shown that the third septum is closed in the majority of wild-type hyphae when mycelium is grown on minimal medium with 1% glucose. Indeed, 7 out of 10 septa separating the third and fourth compartment (when counting from the tip) were closed (Fig. 7A–D; Movies S1 and S2). This was con-

cluded from the fact that cytoplasm of the fourth compartment was not spilled into the environment when the third compartment was cut with a laser. In three cases, the septa were initially open, resulting in some spilling of cytoplasm. Interestingly, in the case of the *spc33* mutant, septa were open in 8 out of 10 cases (Fig. 7E–H; Movies S3 and S4). As a result, the cytoplasm from these 8 hyphae spilled into the medium (see Movies S3 and S4).

Discussion

Septal pore caps were discovered in 1958 as dome-shaped structures that cover the pores of septa of the *Agaricomycotina* (Girbardt, 1958). This group of basidiomycetes encompasses many mushroom-forming fungi. More than 50 years later, the composition and function of the SPCs were still largely unknown. We here purified the SPCs of *S. commune* and show that they consist of a protein matrix surrounded by a lipid layer. One of the proteins that make up the matrix, Spc33, is essential for the formation of the SPC. In the absence of this protein the hyphae could no longer plug the septa after damaging of hyphae. Moreover, growth and mushroom formation were severely affected.

Septal pore caps of *S. commune* were purified from a mycelial homogenate by a combination of treatment with

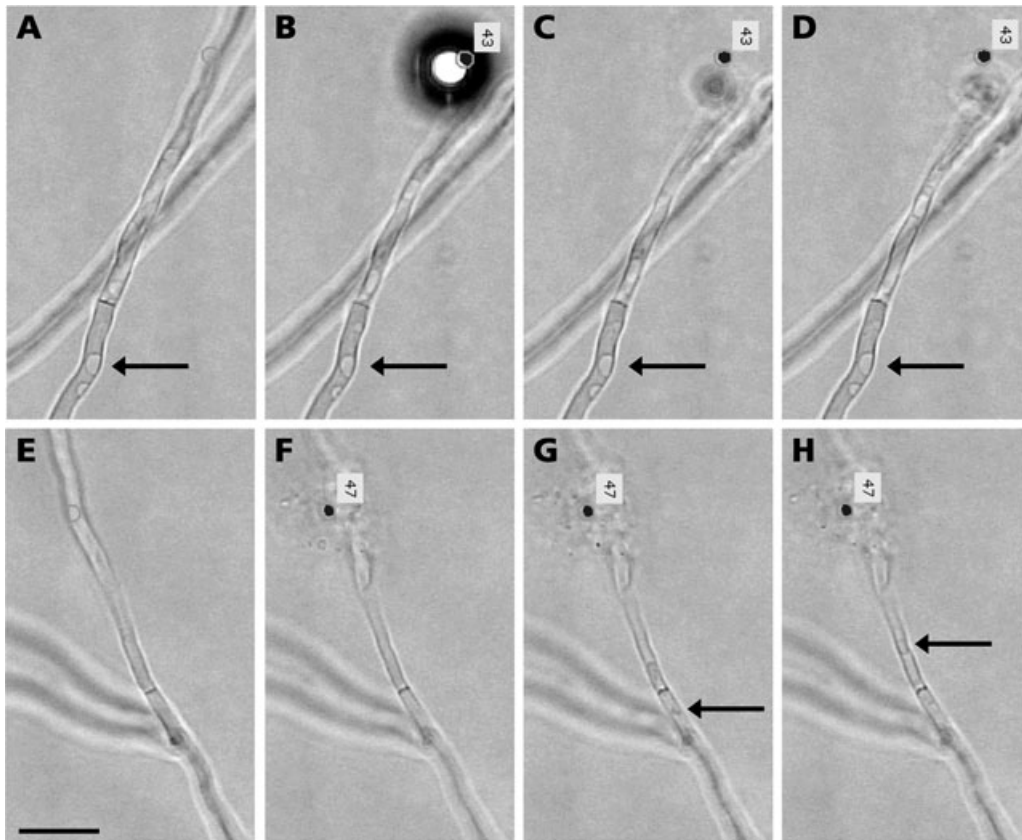


Fig. 7. Laser dissection of a wild-type hypha (A–D) and a hypha of the $\Delta spc33$ strain (E–H). No cytoplasm is spilled from the fourth compartment of the wild-type hypha when the third compartment is damaged by a laser shot (shown in B). This is illustrated by the fact that vacuoles (indicated by an arrow) in the fourth compartment do not move. In contrast, cytoplasm of the fourth compartment of the $\Delta spc33$ strain is rapidly flowing into the environment after a laser shot (shown in F). Vacuoles (indicated by an arrow) rapidly move from the fourth compartment to the third (G–H).

1% Triton X-100, two discontinuous sucrose gradients, and a filtration step over a 220 nm cut-off filter. Triton X-100 dissociated membranous material that otherwise contaminated the SPC fraction. Addition of Triton X-100 also increased the SPC buoyant density from 1.2295 to 1.2575 g cm⁻³. The latter buoyancy correlates with that of pure proteins and protein complexes. Concomitantly with this shift in density, the shape of the pores in the SPC changed from round to hexagonal. These findings are consistent with the idea that membranes represent the outer layers of the SPC (Girbardt, 1958; 1961; Moore and McAlear, 1962; Bracker and Butler, 1963; Marchant and Wessels, 1973; Müller *et al.*, 1998; 2000). The data also show that the inner part of the SPC consists of protein. The proteinaceous matrix still displays the characteristic SPC morphology and thus defines the overall ultrastructure of the organelle.

SDS-PAGE analysis of purified SPC matrix revealed three protein bands with apparent molecular weights of 14, 33 and 60 kDa. The protein with an apparent molecular weight of 14 kDa protein is encoded by *spc14*,

whereas the two other bands are forms of the protein encoded by *spc33*. Genes *spc14* and *spc33* are unique for basidiomycetes that contain SPCs. It should be noted that genomes have only been sequenced from species with perforated SPCs. It would be very interesting when genome sequences of taxa with imperforate and vesiculate SPCs would become available. This would enable us to address whether such taxa contain homologues of *spc14* and *spc33*.

Gene *spc14* encodes a protein of 86 amino acids, which does not have any known domain, signal or localization sequence. From this we postulate that the protein is integrated in the SPC matrix from the cytosol. MPSS and EST analysis showed that *spc33* mRNA is alternatively spliced in *S. commune*. As a result, it encodes a 239- and a 340-amino-acid variant, which share 228 amino acids at their N-termini. Unfortunately, it was not revealed which of the forms is produced. Homologues of *spc33* in *P. placenta*, *P. chrysosporium*, *L. bicolor* and *C. cinerea* also potentially encode the two variants that result from alternative splicing. This and the high

conservation of both forms suggest production of both variants. Both Spc33 variants are predicted to be targeted to the ER because of the sequence at the N-terminus. Signal P predicts that this N-terminal part contains a non-cleavable signal anchor. Indeed, N-terminal sequencing showed that this part of the protein is not spliced. The positive charged amino acids at the N-terminal part predict that this part of the protein resides in the cytosol. The C-terminal part would then reside in the ER lumen. However, according to TMpred in ExPaSy, there is a second transmembrane domain approximately 50 amino acids downstream of the signal anchor. Consequently, both the N-terminus and C-terminus would be exposed in the cytosol, whereas the part in between both transmembrane regions would reside in the ER lumen. It is tempting to speculate that the cytosolic part(s) of Spc33 interacts with Spc14. This would explain why this predicted cytosolic protein is found in the purified SPC fraction. Possibly, Spc33 and Spc14 also form homodimers. This would explain the apparent molecular weight of these proteins in SDS-PAGE assuming that the homodimers resist the presence of SDS, as was observed for hydrophobins (de Vries *et al.*, 1993; Wösten *et al.*, 1993).

A localization of Spc33 in the ER agrees with previous studies that indicate that the SPC is part of this organelle or is derived thereof (Girbardt, 1958; 1961; Moore and McAlear, 1962; Bracker and Butler, 1963; Marchant and Wessels, 1973; Müller *et al.*, 1998; 1999; 2000; van Driel *et al.*, 2008). Immuno-localization confirmed the presence of Spc33 in the SPC of *S. commune* but also showed its absence in 'regular' ER. This was also previously observed for the SPC18 protein of *R. solani* (van Driel *et al.*, 2008). This and the much higher buoyant density of the SPC when compared with ER [1.2295 g cm^{-3} (see above) versus $1.07\text{--}1.14 \text{ g cm}^{-3}$ (Labarère and Bonneau, 1982; Titorenko *et al.*, 1997) respectively] strengthen the idea that the SPC is a specialized structure derived from ER.

Electron microscopy revealed that the SPC was absent in the Δspc33 mutant. As a result, septa did not close after damaging of hyphae and, thus, cytoplasm spilled into the substrate. This, for the first time, shows genetic evidence for a connection between the SPC and plugging of septa. Previously, we could show by immuno-detection that the SPC18 protein of *R. solani* was located both in the SPC and in the plug that closes a septum (van Driel *et al.*, 2008). Taken together, these data indicate that the SPC acts as a repository for plugging material. This mechanism of damage control is different from that found in ascomycetes such as *Neurospora crassa*. In these fungi peroxisome-like organelles, called 'Woronin bodies', plug pores when hyphae become damaged (Markham, 1994; Jedd and Chua, 2000).

Absence of the SPC not only resulted in spilling of cytoplasm upon hyphal damage, it also affected growth and development. Biomass of mono- and dikaryons was reduced by 80% in the Δspc33 mutant and formation of fruiting bodies was severely affected. How can we explain this dramatic phenotype? Our data indicate that the SPC functions by closing the septal pore. It is tempting to speculate that this is a mechanism to locally increase the turgor pressure allowing expansion of vegetative hyphae as well as hyphae that form the aerial reproductive structure. Future studies will address the exact role of the SPC in the lifestyle of mushroom-forming fungi. Possibly, this structure also plays a role in the closure of septa during adverse environmental conditions, during sporulation and during heterokaryon incompatibility. Moreover, it may play a role in the capacity of fungi to colonize large areas, in some cases exceeding 1000 ha of forest (Smith *et al.*, 1992; Ferguson *et al.*, 2003).

Experimental procedures

Strains and growth conditions

Schizophyllum commune wild-type strains 4–40 (MATA₄₃, MATB₄₃, CBS 340.81), 4–39 (MATA₄₁, MATB₄₁, CBS 341.81), 4–8 (MATA₄₃, MATB₄₁, FGSC#9210VT#H4-8) and 4–8b (MATA₄₁, MATB₄₃, University of Utrecht) were used as well as an isogenic derivative of 4–40, in which the *sc3* and *sc15* genes have been deleted (Lugones *et al.*, 2004). Strains were grown in the light at 25°C or 30°C on minimal medium (MM; Dons *et al.*, 1979) solidified with 1.5% agar or as a liquid culture at 225 r.p.m.

Laser dissection of hyphae

Plugging experiments were performed as described (van Peer *et al.*, 2009a). Wild-type and Δspc33 strains were grown in a water vapour saturated chamber at 25°C for 2–3 days in glass bottom culture dishes (P35G-0-20-C, MatTek Corporation, Ashland, MA, USA). Wells in the dishes (20 mm in diameter, 1 mm in height) were filled with 400 μl MM containing 1% agarose. Cultures were inoculated with mycelial plugs that were gently pushed in the agar medium. After inoculation, the agar medium was overlaid with 2 ml liquid MM.

Purification of the septal pore cap

For purification of the SPC the isogenic derivative of 4–40 was used in which the *sc3* and *sc15* genes have been deleted (Lugones *et al.*, 2004). This was done because the abundant cell wall proteins Sc3 (Wösten *et al.*, 1993; 1994) and Sc15 (Lugones *et al.*, 2004) co-purified with the SPCs. A colony of the $\Delta\text{sc3}\Delta\text{sc15}$ strain was grown for 5 days on solid minimal medium topped with a porous polycarbonate membrane (diameter 76 mm, pore size 0.1 μm ; Osmonics, GE Water Technologies, Trevose, PA, USA). The colony was homogenized in a blender (Waring Products, Waring

Laboratory, Torrington, USA) in 100 ml MM. The homogenate was grown for 24 h at 225 r.p.m. This culture was again homogenized and 1 volume of MM was added. After growing for 24 h this procedure was repeated, resulting in 400 ml of densely grown mycelium. Mycelium was separated from the culture medium by filtration over filter paper. 15 g aliquots of mycelium (wet weight) were mixed with 40 ml protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) at 4°C and homogenized in a French Pressure Cell Press (American Instrument Company, Silver Spring, Maryland) in three cycles at 1000 Psi (16 000 cell pressure, high ratio). Cold Triton X-100 (Serva, electrophoresis GmbH, Heidelberg, Germany) was added to a final concentration of 1%, after which the homogenate was incubated for 30 min on ice. 20 ml aliquots were loaded on a discontinuous sucrose gradient consisting of 5.5 ml 55% w/w sucrose, and 5 ml 40% w/w sucrose in Ultra-clear™ Centrifuges tubes (25 × 89 mm, Beckman Coulter, Fullerton, USA). Fractions were collected with a needle connected to a peristaltic pump (LKB Bromma, Bromma, Sweden) after centrifugation for two hours at 100 000 *g* in a SW28 swing out rotor (Beckman Coulter). The SPC containing fraction at the 40–55% sucrose interface was diluted 4 times with 1% Triton X-100 and was loaded on a three-layered sucrose gradient that consisted of 3.5 ml 70% w/w sucrose, 5 ml 52% w/w sucrose, and 5 ml 47% w/w sucrose. The 52–70% interface was isolated after centrifugation for 2 h at 100 000 *g* at 4°C. The fraction was diluted 5 times with cold distilled water and filtered over a 220 nm cut-off pore-size filter (Nitrocellulose filters, Millipore, Billerica, USA) mounted in a Swinnex membrane holder (Millipore). Filters were inverted in the membrane holder and SPCs were washed off with ultra pure water and collected by centrifugation at 10 000 *g* for 60 min.

Protein analysis

SDS-PAGE was performed on 3–18% gradient gels (Bio-Rad Laboratories, Hercules, USA). Proteins were stained with colloidal Coomassie brilliant blue (CBB) (Neuhof *et al.*, 1988) or blotted onto PVDF membranes for immunodetection.

N-terminal sequencing, MS-MS and LC-MS-MS

Proteins were separated on 3–12% gradient SDS-PAA gels containing 40 mM thioglucanate (Bio-Rad Laboratories, Hercules, USA). For N-terminal sequencing (Dr J. Keen; University of Leeds), proteins were blotted onto a PVDF membrane, stained with CBB, and excised. For MS-MS and LC-MS-MS

(Service XS, Leiden, the Netherlands), protein bands were excised from gel after staining with CBB.

Preparation of antiserum

Spc33 Antiserum was produced in rabbits by Sigma Genosys (Sigma-Aldrich, St Louis, MO, USA) by injecting the synthetic peptides EVVNRREHDASRRE and LKVAERERDISKRE.

Western analysis

Proteins were separated by SDS-PAGE, and blotted onto a PVDF membrane. Blots were blocked for 1 h with TBS (18.5 mM Tris, 0.5 M NaCl, pH7) containing 0.05% Tween 20 and 5% Protifar (Nutricia Ltd, Trowbridge, UK). After washing with TBS-Tween, blots were incubated for 1 h with 2000-fold diluted anti-Spc33 in TBS-Tween containing 0.1% Protifar. After washing with TBS, blots were incubated for 1 h with 10 000-fold diluted goat-anti-rabbit-conjugated-alkaline phosphatase (Biosource, Camarillo, CA, USA) in TBS-Tween, 0.1% protifar. Blots were stained with NBT (0.5 mg ml⁻¹) and BCIP (1.25 mg ml⁻¹) (Sigma-Aldrich) in 10 ml alkaline phosphatase buffer (0.1 M Tris, 0.1 M NaCl and 5 mM MgCl₂, PH 9.5).

Cloning of the *spc33* knock-out construct

Vector pDELCS (van Peer, 2008) was used to inactivate *spc33*. This vector contains a phleomycin and a nourseothricin resistance cassette. Flanking regions of *spc33* gene were cloned at either site of the nourseothricin resistance cassette making use of restriction sites for Van911 and Sfil. Digestion of pDELCS with Van911 generates two different sticky ends that are compatible with sequences in the primers SPC33KO1 and SPC33KO2 (Table 2), thus allowing directional cloning of fragments amplified with these primers. Likewise, the Sfil sites generate two different overhangs upon digestion that are compatible with sequences in the primers SPC33KO3 and SPC33KO4 (Table 2). The 1434 bp upstream flank and the 1659 bp downstream flank of the coding sequence of *spc33* were amplified by PCR using *S. commune* chromosomal DNA as a target and primer sets SPC33KO1 and SPC33KO2 and SPC33KO3 and SPC33KO4 respectively. The PCR fragments were cloned into pGEMTeasy, resulting in vectors pAVP8 (*spc33* upstream flank) and pAVP9 (*spc33* downstream flank). The upstream flank was excised with Sfil and introduced in the Van911 site

Table 2. Primers used in this study.

Primer	Primer sequence	Notes
SPC33KO1	GGCCTAATAGGCTGGAAGCCGACCAAG	Introduces Van911 compatible site
SPC33KO2	GGCCTCGCAGGCCATCCTGACGCTGCTGTAG	Introduces Van911 compatible site
SPC33KO3	GGCCTGCGAGGCCTGTGGCTTGCCAATGTTATG	Introduces Sfil compatible site
SPC33KO4	GGCCTATTAGGCTCAGGATGGTCCCGTCTTG	Introduces Sfil compatible site
SC3tersqf	GCCTCAGGTCCCCGAAGTAAG	
SPC33dfr3	GGCCTATTAGGCCCTGTCCGATGCACAGTGATG	
SPC33fw1	GGATGGCCCAACCTTCCAC	
SPC33rv1	CAGCGTCCCGAGACGACTGAAAAG	

of pDELICAS, resulting in pAVPKO33a. The downstream flank was excised from pAVP9 with SfiI and cloned into pAVPKO33a, which had been cut with the same enzyme. This resulted in the *spc33* knock-out construct pAVPKO33b.

Transformation of *S. commune*

Transformation of *S. commune* strain 4–8 was done according to van Peer and colleagues (2009b). Regeneration medium (MM + 0.5 M MgSO₄) and selection plates contained 10 µg ml⁻¹ nourseothricin. Plates were incubated at 30°C for 5 days.

Isolation of genomic DNA and RNA and MPSS expression analysis

DNA isolation and hybridizations were done as described (Schuren *et al.*, 1993; van Peer *et al.*, 2009b). RNA was isolated according to van Peer and colleagues (2009b) and purified with an RNA clean-up kit (Macherey-Nagel, Germany). Quality of the RNA was analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). MPSS was performed essentially as described (Brenner *et al.*, 2000) except that after DpnII digestion Mmel was used to generate 20 bp tags (Meyers *et al.*, 2004). Tags were sequenced using the Clonal Single Molecule Array technique (Illumina, Hayward, CA, USA).

Screening for homologous integration in *S. commune*

Strains in which the deletion construct pAVPKO33b was introduced were selected for nourseothricin resistance (first screen) and phleomycin sensitivity (second screen). Chromosomal DNA was isolated from knockout candidates and analysed by PCR. Presence of an intact *spc33* gene was screened for with the primer pair SPC33fw1 and SPC33rv1 (Table 2). Presence of the deletion of *spc33* was shown by using the primer pair SC3tersqf and SPC33dfr3 (Table 2). Primer SC3tersqf anneals to the SC3 terminator directly downstream of the nourseothricin resistance, whereas primer SPC33dfr3 anneals downstream of the 3' end of the downstream flank of *spc33*. This primer pair will only give a product in the case of homologous integration.

Chemical fixation and freeze substitution of *S. commune* mycelium

Mycelium was fixed overnight at 4°C in 1.5% glutaraldehyde (Agar Scientific LTD, Essex, UK) buffered with 20 mM HEPES, pH 6.8. After post-fixation in 1% (w/v) aqueous osmium tetroxide (EMS, Hatfield, PA, USA) for 60 min, samples were washed with ultra-pure water twice, and gradually dehydrated in 60% (v/v), 70% (v/v), 80% (v/v), 90% (v/v) and 100% (two times) acetone for 30 min each. After taking up the material in acetone containing 1% (v/v) acidified 2,2-dimethoxypropane (DMP), samples were infiltrated stepwise (each step for 2 h) with 25% (v/v), 50% (v/v), 75% (v/v) and 100% (two times) Spurr's resin (Spurr, 1969). After washing with freshly prepared Spurr's resin, samples were embedded

in the resin at 65°C for 48 h in BEEM capsules (EMS). Sections were cut with a diamond knife (Diatome, Hatfield, PA, USA) using a ULTRACUT E ultramicrotome (Leica Microsystems, Vienna, Austria). Sections were picked up with formvar-coated, carbon-stabilized copper grids (hexagonal 150 mesh Veco grids, EMS). Sections were contrasted with 4% (w/v) aqueous uranyl acetate (Merck, Darmstadt, Germany) for 10 min and 0.4% (w/v) aqueous lead citrate (Merck) for 2 min (Venable and Coggeshall, 1965).

For high pressure freezing and freeze-substitution, peripheral colony parts (2 mm diameter) were cut with an eye biopsy punch (Grieshaber, Switzerland) and put into 100 µm aluminum planchets. Planchets were closed with back-sides of 300 µm aluminum planchets and high-pressure frozen in a holder from Leica. Prior to use, planchets were coated with 10% lecithin (Fluka, Sigma-Aldrich) in ethanol (w/v) to enable easy separation under liquid nitrogen. After high-pressure freezing, samples were opened under liquid nitrogen and colony parts (still attached to the 100 µm planchets) were transferred to a 2.0 ml Saf-T-seal free-standing tube (Biozyme) with substitution medium (0.3% uranyl acetate and 0.01% glutaraldehyde in anhydrous methanol). Tubes were placed into a CS-auto substitution apparatus (Sitte *et al.*, 1985) at -90°C for 48 h, after which the temperature was raised to -40°C at a speed of 3°C h⁻¹. The freeze-substitution medium was exchanged for methanol and samples were infiltrated with increasing concentrations of Lowicryl mixture containing K11M: HM20 (3:1) and 0.05% uranyl acetate (Carlemalm *et al.*, 1982; Edelmann and Ruf, 1996). Samples were infiltrated with 25% mixture for 2 h, 50% for 2 h, 75% for 15 h, 100% for 1 h, 100% for 72 h, 100% for 48 h, and fresh made 100% Lowicryl mixture for 48 h. Samples were embedded in 100% Lowicryl mixture and polymerized with UV at -40°C for 48 h (Carlemalm *et al.*, 1982) and 1 day of curing under UV at room temperature. Ultrathin sections were blocked for 5 min in 5% foetal calf serum (PAA laboratories GmbH, Pasching, Austria) in PBS, washed with 1% BSA in PBS, and labelled with anti-Spc33 antibodies (1:250) and protein A-10 nm gold (1:55) (Department of Cell Biology, UMC, Utrecht, the Netherlands) in PBS containing 1% BSA. Samples were washed with PBS containing 0.1% BSA in between the incubation steps. The gold-labelled sections were post-fixed with 1% glutaraldehyde in PBS for 3 min and post-stained for 5 min as described above.

Transmission electron microscopy

Samples were observed with a TECNAI 10 (FEI Company, Hillsboro, OR, USA) electron microscope at an acceleration voltage of 100 kV.

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References

Barr, D.J.S. (2001) Chytridiomycota. In *The Mycota VII, Systematics and Evolution, Part A*. McLaughlin, D.J.,

- McLaughlin, E.G., and Lemke, P.A. (eds). Berlin, Germany: Springer-Verlag, pp. 93–112.
- Bendtsen, J.D., Nielsen, H., von Heijne, G., and Søren Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**: 783–795.
- Benny, G.L., Humber, R.A., and Morton, J.B. (2001) Zygomycota: Zygomycetes. In *The Mycota VII, Systematics and Evolution, Part A*. McLaughlin, D.J., McLaughlin, E.G., and Lemke, P.A. (eds). Berlin, Germany: Springer-Verlag, pp. 113–146.
- Berteaux-Lecellier, V., Zickler, D., Debuchy, R., Panvier-Adoutte, A., Thompson-Coffe, C., and Picard, M. (1998) A homologue of the yeast SHE4 gene is essential for the transition between the syncytial and cellular stages during sexual reproduction of the fungus *Podospora anserina*. *EMBO J* **17**: 1248–1258.
- Blom, N., Gammeltoft, S., and Brunak, S. (1999) Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* **294**: 1351–1362.
- Bracker, C.E., and Butler, E.E. (1963) The ultrastructure and development of septa in hyphae of *Rhizoctonia solani*. *Mycologia* **55**: 35–58.
- Bracker, C.E., and Butler, E.E. (1964) Function of the septal pore apparatus in *Rhizoctonia solani* during protoplasmic streaming. *J Cell Biol* **21**: 152–157.
- Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D.H., Johnson, D., et al. (2000) Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat Biotechnol* **18**: 630–634.
- Buller, A.H.R. (1933) The translocation of protoplasm through the septate mycelium of certain Pyrenomycetes, Discomycetes, and Hymenomycetes. In *Researches on Fungi*, Vol. 5. Buller, A.H.R. (ed.). New York, USA: Hafner Publishing, pp. 75–167.
- Carlemalm, E., Garavito, R.M., and Villiger, W. (1982) Resin development for electron microscopy and an analysis of embedding at low temperature. *J Microsc* **126**: 123–143.
- Claros, M.G., and von Heijne, G. (1994) TopPred II: an improved software for membrane protein structure predictions. *CABIOS* **10**: 685–686.
- Dons, J.J.M., de Vries, O.M.H., and Wessels, J.G.H. (1979) Characterization of the genome of the basidiomycete *Schizophyllum commune*. *Biochim Biophys Acta* **563**: 100–112.
- van Driel, K.G.A., van Peer, A.F., Grijpstra, J., Wösten, H.A.B., Verkleij, A.J., Müller, W.H., and Boekhout, T. (2008) Septal pore cap protein SPC18, isolated from the basidiomycetous fungus *Rhizoctonia solani*, also resides in pore plugs. *Eukaryot Cell* **7**: 1865–1873.
- van Driel, K.G.A., Humbel, B.M., Verkleij, A.J., Stalpers, J., Muller, W.H., and Boekhout, T. (2009) Septal pore complex morphology in the agaricomycotina (basidiomycota) with emphasis on the cantharellales and hymenochaetales. *Mycol Res* (Epub ahead of print).
- Edelmann, L., and Ruf, K. (1996) Freeze-dried human leukocytes stabilized with uranyl acetate during low temperature embedding or with OsO₄ vapor after embedding. In *Scanning Microscopy Supplement 10*. O'Hare, A.M.F. (ed.). Chicago, IL, USA: Scanning Microscopy International, pp. 295–307.
- Ferguson, B.A., Dreisbach, T.A., Parks, C.G., Filip, G.M., and Schmitt, C.L. (2003) Coarse-scale population structure of pathogenic *Armillaria* species in a mixed conifer forest in the Blue Mountains of northeast Oregon. *Can J For Res* **33**: 612–623.
- Girbardt, M. (1958) Über die Substruktur von *Polystictus versicolor* L. *Arch Mikrobiol* **28**: 255–269.
- Girbardt, M. (1961) Licht- und Elektronenmikroskopische Untersuchungen an *Polystictus versicolor*. *Arch Mikrobiol* **39**: 351–359.
- Glass, N.L., and Kaneko, I. (2003) Fatal attraction: non-self recognition and heterokaryon incompatibility in filamentous fungi. *Eukaryot Cell* **2**: 1–8.
- von Heijne, G. (1992) membrane protein structure prediction: hydrophobicity analysis and the 'positive inside' rule. *J Mol Biol* **225**: 487–494.
- James, T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V., Cox, C.J., et al. (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* **443**: 818–822.
- Jedd, G., and Chua, N.H. (2000) A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane. *Nat Cell Biol* **2**: 226–231.
- Labarère, J., and Bonneau, M. (1982) Isolation and characterization of plasma membranes from the fungus *Podospora anserina*. *J Bacteriol* **151**: 648–656.
- Lugones, L.G., de Jong, J.F., de Vries, O.M.H., Jalving, R., Dijksterhuis, J., and Wösten, H.A.B. (2004) The SC15 protein of *Schizophyllum commune* mediates formation of aerial hyphae and attachment in the absence of the SC3 hydrophobin. *Mol Microbiol* **53**: 707–716.
- McLaughlin, D.J., Frieders, E.M., and Lü, H. (1995) A microscopist's view of hetero-basidiomycete phylogeny. *Stud Mycol* **38**: 91–109.
- Marchant, R., and Wessels, J.G.H. (1973) Septal structure in normal and modified strains of *Schizophyllum commune* carrying mutations affecting septal dissolution. *Arch Microbiol* **90**: 35–45.
- Markham, P. (1994) Occlusion of septal pores in filamentous fungi. *Mycol Res* **8**: 1089–1106.
- Meyers, B.C., Tej, S.S., Vu, T.H., Haudenschild, C.D., Agrawal, V., Edberg, S.B., et al. (2004) The use of MPSS for whole-genome transcriptional analysis in Arabidopsis. *Genome Res* **14**: 1641–1653.
- Moore, R.T., and McAlear, J.H. (1962) Fine structures of mycota. Observations on septa of ascomycetes and basidiomycetes. *Am J Bot* **49**: 86–94.
- Moore, R.T., and Patton, A.M. (1975) Parenthesome fine structure in *Pleurotus cystidiosus* and *Schizophyllum commune*. *Mycologia* **67**: 1200–1205.
- Müller, W.H., van Aelst, A.C., van der Krift, T.P., and Boekhout, T. (1994) Scanning electron microscopy of the septal pore cap of the basidiomycete *Schizophyllum commune*. *Can J Microbiol* **40**: 879–883.
- Müller, W.H., Montijn, R.C., Humbel, B.M., van Aelst, A.C., Boon, E.J., van der Krift, T.P., and Boekhout, T. (1998) Structural differences between two types of basidiomycete septal pore caps. *Microbiology* **144**: 1721–1730.
- Müller, W.H., Humbel, B.M., van Aelst, A.C., van der Krift, T.P., and Boekhout, T. (1999) The perforate septal pore cap of basidiomycetes. In *Plasmodesmata. Structure, Func-*

- tion, *Role in Cell Communication*. van Bel, A.J.E., and van Kesteren, W.J.P. (eds). Berlin, Germany: Springer-Verlag, pp. 120–127.
- Müller, W.H., Koster, A.J., Humbel, B.M., Ziese, U., Verkleij, A.J., van Aelst, A.C., et al. (2000) Automated electron tomography of the septal pore cap in *Rhizoctonia solani*. *J Struct Biol* **131**: 10–18.
- Neuhof, V., Arold, N., Taube, D., and Erhardt, W. (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**: 255–262.
- Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10**: 1–6.
- van Peer, A.F. (2008) Structure and function of the septal pore cap of *Schizophyllum commune*. PhD Thesis. Utrecht, The Netherlands: University of Utrecht.
- van Peer, A.F., de Bekker, C., Vinck, A., Wösten, H.A.B., and Lugones, L.G. (2009b) Phleomycin increases transformation efficiency and promotes single integrations in *Schizophyllum commune*. *Appl Environ Microbiol* **75**: 1243–1247.
- van Peer, A.F., Müller, W.H., Boekhout, T., Lugones, L.G., and Wösten, H.A.B. (2009a) Cytoplasmic continuity revisited: closure of septa of the filamentous fungus *Schizophyllum commune* in response to environmental conditions. *PLoS One* **4**: e5977.
- Schuren, F.H.J., Harmsen, M.C., and Wessels, J.G.H. (1993) A homologous gene-reporter system for the basidiomycete *Schizophyllum commune* based on internally deleted homologous genes. *Mol Gen Genet* **238**: 91–96.
- Sitte, H., Neumann, K., and Edelmann, L. (1985) Cryofixation and cryosubstitution for routine work in transmission electron microscopy. In *Science of Biological Specimen Preparation*. Müller, M., Becker, R.P., Boyde, A., and Wolosewick, J.J. (eds). Chicago, IL, USA: SEM, pp. 103–108.
- Smith, M.L., Bruhn, J.N., and Anderson, J.B. (1992) The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. *Nature* **356**: 428–431.
- Spurr, A.R. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* **26**: 31–43.
- Titorenko, V.I., Ogrydziak, D.M., and Rachubinski, R.A. (1997) Four distinct secretory pathways serve protein secretion, cell surface growth, and peroxisome biogenesis in the yeast *Yarrowia lipolytica*. *Mol Cell Biol* **17**: 5210–5226.
- Venable, J.H., and Coggeshall, R. (1965) A simplified lead citrate stain for use in electron microscopy. *J Cell Biol* **25**: 407–408.
- de Vries, O.M.H., Fekkes, M.P., Wösten, H.A.B., and Wessels, J.G.H. (1993) Insoluble hydrophobin complexes in the walls of *Schizophyllum commune* and other filamentous fungi. *Arch Microbiol* **159**: 330–345.
- Wösten, H.A.B., de Vries, O.M.H., and Wessels, J.G.H. (1993) Interfacial self-assembly of a fungal hydrophobin into a rodlet layer. *Plant Cell* **5**: 1567–1574.
- Wösten, H.A.B., Ásgeirsdóttir, S.A., Krook, J.H., Drenth, J.H.H., and Wessels, J.G.H. (1994) The fungal hydrophobin Sc3p self-assembles at the surface of aerial hyphae as a protein membrane constituting the hydrophobic rodlet layer. *Eur J Cell Biol* **63**: 122–129.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PCR with genomic DNA of a strain in which vector pAVPKOSPC33b has been introduced (lanes 2, 4) and with DNA of a wild-type strain (lanes 3, 5) using primers that anneal to the downstream flank of the nourseotricine cassette and the 3' genomic region of the downstream flank (lanes 2, 3) and that anneal to the 5' and 3' end of the coding sequence of *SPC33* (lanes 4, 5). From this it is concluded that the transformant has a deletion in the *SPC33* gene. Lane 1 represents a DNA standard.

Movies S1–S4. Laser dissection of wild-type hyphae (Movies S1, S2) and hyphae of the Δ SPC33 strain (Movies S3, S4). No cytoplasm is spilled from the fourth compartment of wild-type hyphae when the third compartment is damaged by a laser shot. This is illustrated by the fact that vacuoles in the fourth compartment do not move. In contrast, cytoplasm of the fourth compartment of the Δ SPC33 strain is rapidly flowing into the environment after a laser shot (Movies S3, S4).

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