A hydrophobin (ABH3) specifically secreted by vegetatively growing hyphae of *Agaricus bisporus* (common white button mushroom)

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Aerial mycelium and hyphal strands of *Agaricus bisporus*, strain U1, exhibited a rodlet pattern at their surfaces characteristic for assembled class I hydrophobins. An SDS-insoluble/trifluoroacetic-acid-soluble fraction from strands was found to contain one abundant protein with an apparent molecular mass on gel of 19 kDa. Two sequences for this protein (ABH3), typical of class I hydrophobins, could be deduced by sequencing cDNA clones obtained by RT-PCR. The two forms of the protein could be assigned to different alleles present in the two homokaryons that constitute the heterokaryotic U1 strain. ABH3 displays all the in vitro properties of a typical class I hydrophobin such as SC3 from *Schizophyllum commune* but is not glycosylated or otherwise post-translationally modified because the molecular mass values deduced from the amino acid sequence (9228 and 9271 Da) and derived from mass spectrometry were in good agreement. The ABH3 transcript was found to be present in the vegetative mycelium of both primary and secondary mycelium but not in the fruiting bodies, whereas the reverse was found for the ABH1 hydrophobin. Using an *S. commune* mutant with a disrupted SC3 gene it was found that ABH3 can substitute for SC3 in inducing formation of aerial hyphae, suggesting a role of ABH3 in the emergence of aerial hyphae and strands in *A. bisporus*.

Keywords: *Agaricus bisporus*, hydrophobin, wall protein, substrate mycelium

INTRODUCTION

Hydrophobins are small, moderately hydrophobic proteins secreted by fungi and characterized by a conserved spacing of eight cysteine residues and by a typical hydrophathy pattern (Wessels, 1994). While a large number of sequences putatively encoding such proteins have been found in various fungi, only in a few cases has the protein itself been isolated and characterized (Wessels, 1997). Those that have been studied exhibit the remarkable property of self-assembly into amphipathic films at hydrophobic–hydrophilic interfaces. On the basis of solubility of these assemblages, two hydrophobin classes were distinguished (Wessels, 1994). Class I hydrophobins, exemplified by the SC3 hydrophobin of *Schizophyllum commune* (Schuren & Wessels, 1990), form very stable SDS-insoluble protein films with a typical rodlet pattern at the hydrophobic face (Wessels *et al.*, 1991; Wosten *et al.*, 1993, 1994a). Class II hydrophobins, exemplified by cerato-ulmin from *Ophiostoma ulmi* (Bowden *et al.*, 1994), form unstable SDS-soluble films (Richards, 1993).

The SC3 hydrophobin of *S. commune* is known to assemble at the surface of aerial hyphae and to cover these with a hydrophobic rodlet layer, making these hyphae extremely hydrophobic (Wosten *et al.*, 1993, 1994b). Disruption of the SC3 gene caused formation of hydrophilic aerial hyphae and, under certain conditions, prevented formation of any aerial hyphae (Wosten *et al.*, 1994a; van Wetter *et al.*, 1996). However, SC3 affects formation of aerial hyphae not only by conferring hydrophobicity to aerial hyphae but probably also enables substrate hyphae to breach the medium–air interface by causing a large drop in the surface tension of the medium (van der Vegt *et al.*, 1996; H. A. B. Wösten, M.-A. van Wetter & J. G. H. Wessels, unpublished data).
Previously, a fruiting-body-specific hydrophobin called ABH1 (Lugones et al., 1996) or HYP A (de Groot et al., 1996) was reported to occur in Agaricus bisporus. It forms a hydrophobic rodlet layer at the surface of the fruiting bodies and also lines the air channels within fruiting bodies (Lugones et al., 1996). A second hydrophobin gene was found 1-8 kb downstream of the ABH1 gene (ABH2/hypC) which is expressed in fruiting bodies at a very low level (de Groot et al., 1996; Lugones et al., 1996). de Vries et al. (1993) reported the secretion of a class-I-hydrophobin-like protein by the substrate mycelium of A. bisporus which could not be identical to that encoded by the ABH1 or ABH2 genes based on the expression data.

Here we report the isolation and characterization of a class-I-hydrophobin (ABH3) secreted by the substrate mycelium of Agaricus bisporus, which could not be identical to that encoded by the ABH1 or ABH2 genes based on the expression data.

METHODS

Organism and culture conditions. Agaricus bisporus homokaryons Horst 39 and Horst 97 and the derived heterokaryon Horst U1 were obtained from the collection of the Mushroom Experimental Station (Horst, The Netherlands).

Substrate mycelium was obtained in two ways: by growing a homogenate of mycelium in plastic 9 cm Petri dishes containing 25 ml liquid Schizophyllum commune minimal medium (MM; Dons et al., 1979) at 24 °C for 10 d or by inoculating a small piece of substrate mycelium on MMP medium (1% malt extract, 0.5% mycelial peptone, 1.5% agar) or on compost medium (MMP+2% sterilized, dried and ground compost, 1.5% agar). For immunolabelling, a 0.15 cm² piece of colonized agar medium from a full-grown agar plate was put directly on the bottom of an empty polystyrene Petri dish, allowing the mycelium to grow over the plastic at 24 °C for 2 weeks. To prevent desiccation, a ring of sterile filter paper wetted with sterile water was put around the colony and the dish was wrapped in plastic foil. Young A. bisporus (cultivar U1) mushroom primordia measuring 10 mm in diameter (pinheads) were kindly provided by Mr B. Boer (Mushroom Farm Agarica BV, Hoogeveen, The Netherlands). After picking they were immediately processed or frozen in liquid nitrogen and stored at −81 °C. The SC3-disrupted strain of S. commune, a similar role for ABH3 in A. bisporus is envisaged.

Isolation and purification of ABH3. For isolation of the hydrophobin from hyphal walls, frozen substrate mycelium was passed through an X-press (AB Biotec). The material was further treated as described by Lugones et al. (1996). For isolation of the hydrophobin from the medium, the culture filtrate of 1-month-old cultures in MM was subjected to bubbling with gas. To this end, hydrogen gas bubbles were generated from the medium by electrolysis at 100 mA for 3 h. The cathode was kept deep in the liquid and the anode was separated from the medium by a salt bridge. A magnetic stirrer was used to distribute the tiny hydrogen bubbles evenly through the medium, where they were coated by the assembling hydrophobin. Coated hydrogen vesicles floated to the surface and formed a foam that could easily be collected. After lyophilization, material in the foam was treated successively with trifluoroacetic acid (TFA) and dissolved in 60% (v/v) ethanol as described by Lugones et al. (1996). After centrifugation the supernatant was dialysed against water and reaggregated on electrophoretically released hydrogen bubbles. After TFA treatment the ABH3 in the lyophilized material was found to be electrophoretically pure after silver staining.

Protein analysis. Gel electrophoresis and staining, Western blotting, N-terminal sequencing and immunostaining as well as periodic acid–Schiff staining were performed as described by Lugones et al. (1996). MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time of Flight) mass spectroscopy was done by drying 1 µl of a solution of ABH3 (100 µg ml⁻¹) on a target on top of which 1 µl matrix [20 mg sinapinic acid ml⁻¹ in acetonitrile/0.1% TFA in water (40:60, v/v)] was dried. The spectra were recorded in a TofSpec E & SE Micromass mass spectrometer.

Preparation of antiserum against ABH3. Rabbits were injected five times at 10 d intervals with a mixture of 400 µg gel-purified monomeric ABH3 and 400 µg assemb underestimated ABH3. On Western blots, the recovered antiserum reacted specifically with ABH3 and not with other A. bisporus proteins. Preimmune serum was unreactive in all cases. When required for immunolocalization studies, the antiserum (diluted 1:100) was purified by incubating it three times for 60 min with a 1:1 (w/w) mixture of cell walls (Sietetsma et al., 1977) from a fruiting dikaryon of S. commune and from fruiting bodies of A. bisporus at 1 mg (ml antiserum)⁻¹.

RNA isolation. Frozen mycelium was ground to a powder under liquid nitrogen in a mortar and RNA was extracted by the hot-phenol procedure (Wessels et al., 1987).

Northern and Southern blotting. These were performed as described in Schuren et al. (1993). For RNA electrophoresis, equal amounts of RNA were loaded based on A₂₆₀ measurements. The presence of equal amounts was also confirmed by considering the intensity of the ribosomal bands (fluorescence of bound ethidium bromide). When quantification was required, hybridization with an 18S ribosomal probe was also done.

cDNA clone isolation. First-strand synthesis was done in a 50 µl reaction mixture containing 2 µl total RNA extract, 1 U avian myeloblastosis virus reverse transcriptase (Boehringer), 200 µM of each of the four deoxyribonucleotides, 290 nM oligo-dT (26-mer) and 1 µl RNA-guard (Pharmacia) in 1 × RT buffer (supplied with the enzyme). Incubation was for 1 h at 55 °C. After 2 min denaturation at 94 °C, an aliquot (2 µl) was taken for PCR. Reaction mixtures for PCR (50 µl) contained 2.5 U Taq polymerase (Boehringer) and buffer (supplied by the manufacturer), 200 µM of each deoxyribonucleotide, 0.3 µM oligo-dT (26-mer with an additional XhoI restriction site) as reverse primer and 3 µM degenerate primer based on the determined N-terminal amino acid sequence. A moderately stringent annealing profile (1 min, 55 °C) was used. Denaturation was at 94 °C for 1 min and elongation was at 72 °C for 1.5 min. For the isolation of a complete cDNA clone, RACE-PCR was performed (5′/3′ RACE kit; Boehringer) following the protocol recommended by the manufacturer.

Cloning and DNA sequencing. ABH3 cDNA was cloned in pUC vectors or derivatives. Nucleotide sequences were determined by sequencing from both strands with the T7 DNA
Hydropophin ABH3 of Agaricus bisporus

RESULTS

Isolation of ABH3

Surface replicas of mycelial cords and aerial hyphae from A. bisporus U1 were viewed in the electron microscope (Fig. 1a). A mosaic pattern of parallel 10-nm-wide rodlets was observed which resembled that formed by ABH1 on hyphae at the outer surface of fruiting bodies (Lugones et al., 1996). Since ABH1 is not produced by vegetative mycelium (Lugones et al., 1996; de Groot et al., 1996), this rodlet layer probably derives from another hydrophobin and was therefore analysed further. Extraction of a hot-SDS-insoluble residue from cell walls of mycelial cords with TFA solubilized a single protein which upon SDS-PAGE ran at 19 kDa (Fig. 2, lane 1). N-terminal amino acid sequencing showed the sequence (D)TDPPATGSSXTAVGDVNNXXSNL, in which 'X' indicates a possible cysteine residue. As indicated, two forms of the protein were found: one beginning with aspartic acid and another starting with threonine.

When A. bisporus was grown for a few days in static liquid minimal medium a thin film could be discerned on the surface of the medium. By injecting air under this film, air bubbles formed that collapsed and left wrinkles of corrugated film. This material could be easily collected and was freeze-dried. After treatment with TFA, SDS-PAGE revealed a single 19 kDa band (not shown). The N-terminal amino acid sequence of this protein (called ABH3) showed identity with that of the cell wall protein referred to above.

An antibody raised against medium ABH3 reacted with the protein extracted from cell walls on Western blots (data not shown). Staining for carbohydrate was negative, suggesting the absence of glycosylation.

Extraction of the ABH3 hydrophobin from large quantities of medium could be done essentially as described for SC3 hydrophobin from S. commune (Wosten et al., 1993). However, higher yields were obtained if gas bubbles were generated by electrolysis of the medium. In this way, smaller gas bubbles were formed and ABH3 could be separated by flotation instead of centrifugation, also simplifying the isolation procedure. Using this procedure, up to 2 mg pure ABH3 (l medium)-1 could be isolated from a 30-d-old culture.

Isolation and sequencing of the ABH3 cDNA

The primary structure of the protein was obtained from an ABH3 cDNA clone isolated by RT-PCR. For this, a degenerate primer was designed based on the amino acid residues 17-23 of the N-terminal sequence (underlined in the N-terminal sequence given above), assuming the non-identified amino acids (X) to be cysteine residues: S'-GGTGACG(T/C)AA(C/T)TG(C/T)TG(T/C)-AA. To minimize the degeneracy, only the codons mostly used in A. bisporus (van der Vlugt et al., 1993) were considered. After RT-PCR with this forward primer and a reverse primer containing oligo-dT18, a band of 430 bp could be detected on gel which was cloned and sequenced. The deduced amino acid sequence of this cDNA clone identified ABH3 as a class I hydrophobin (Fig. 3). To amplify the missing part of the coding sequence upstream of the forward primer S' RACE-PCR was performed. For this, specific reverse polymerase kit (Pharmacia) using the dideoxy chain-termination method (Sanger et al., 1977).

Interfacial self-assembly. ABH3 was assembled on polytetrafluoroethylene (Teflon) discs (0.5 cm diameter, 0.5 mm thick) which were first cleaned in KNO3-containing hot H2SO4 followed by extensive washes with water and ethanol and then incubated with an aqueous ABH3 solution (10 μg ml-1) for 16 h. The discs were subsequently extracted with 1% SDS at 100°C, washed in distilled water, dried in air, and the hydrophobicity of the surface was measured by determining water contact angles (van der Mei et al., 1991). For assembly of ABH3 on a hydrophilic surface, a solution of the hydrophobin (0.2 mg ml-1) in 35% (v/v) aqueous ethanol was allowed to ascend in a filter paper strip (Schleicher & Schuell no. 0860; 20 × 0.6 cm) in the air. After 18 h, allowing for evaporation of about 2 ml solvent, the paper strip was dried and extracted with 1% SDS at 100°C for 10 min, washed with water and dried for measurement of water contact angles.

Surface tension measurement. Surface tension was determined by Axi-Symmetric Drop Shape Analysis by Profile as described by Noordmans & Busscher (1991). One-hundred microliters of freshly prepared aqueous protein solution (100 μg ml-1) was placed on a cleaned fluoroethylene/propylene/Teflon surface (FEP; Norton Fluorplast). The droplet profile was digitized with a contour monitor and the data were used to calculate the liquid surface tension. All measurements were done at room temperature at least in duplicate, taking 96 profiles of the water droplet over a period of 8 h. To prevent evaporation, measurements were done with the droplet sitting in a water-vapour-saturated chamber.

Immunolocalization of ABH3. Mycelium grown on the bare surface of polystyrene dishes was fixed while attached to the surface of the substrate mycelium using Pt/C and cleaned in KN03-containing hot water and dried for measurement of water contact angles. In a water-vapour-saturated chamber.

Electron microscopy. For visualization of rodlets on the hydrophobic side of assembled ABH3, 5 μl of a solution of ABH3 (approx. 5 μg ml-1) was allowed to dry on Formvar-coated nickel grids. Replicas were made directly from the substrate of the mycelium using Pt/C and cleaned in K3[Cr2(OF)7] saturated H2SO4 for 90 min. Surface shadowing with Pt/C was done at an angle of 45°. Shadowed preparations were examined in a Philips CM 10 electron microscope. Photographs were made on FGP Kodak film.

RESULTS

Isolation of ABH3

Surface replicas of mycelial cords and aerial hyphae from A. bisporus U1 were viewed in the electron microscope (Fig. 1a). A mosaic pattern of parallel 10-
primers were designed based on the obtained partial sequence. A band of 420 bp could be amplified, cloned and sequenced, revealing the sequence upstream of the reverse primers, including the sequence known from N-terminal amino acid sequencing (25 residues) (Fig. 3). An additional segment of 166 bp upstream of the sequence encoding the first amino acid (threonine) of the mature protein contained a putative start codon followed by a 25-residues-long putative signal sequence for secretion in-frame with the rest of the encoded protein.

Sequencing of a number of cDNA clones revealed the presence of two sequences differing at 15 positions which resulted in conservative substitutions in only five amino acids (Fig. 3). This was interpreted as a possible allelism inherent in the heterokaryotic condition of U1. When an ABH3 probe (a 1:1 mixture of the two forms of RACE-PCR products found) was used on genomic DNA from U1 and from the homokaryotic strains derived from it (H39 and H97), multiple bands were observed (result not shown). Even when restriction was done with enzymes with no target within the probe or when a double restriction was done using an enzyme cutting within the probed region and another cutting outside, multiple bands arose in all three strains examined. This raised the possibility of multiple homologous genes besides the possibility of allelism.

Complete ABH3 cDNAs from strains H39 and H97 were amplified by RT-PCR on total RNA from these
Hydrophobin ABH3 of *Agaricus bisporus*

Fig. 4. Hydropathy plot of ABH3, ABH1 and SC3. The sequences have been aligned at the first, second, fourth, fifth and eighth cysteine residue (vertical lines). The hydropathy values were calculated following Kyte & Doolittle (1982) using a window size of six amino acids.

strains. A primer was used based on the sequence around the start codon (common to both forms of ABH3), together with the oligo-dT primer. Five different clones of the amplified band were sequenced for each strain. Within each group the sequences were found to be identical, but between the groups they differed as shown for the two sequences derived from the heterokaryon U1 (Fig. 3). These two sequences thus probably represent different alleles of ABH3 in the homokaryons constituting strain U1 but possibly present in multiple copies in each of these strains. If these copies deviate from each other, only one is being expressed.

Both complete ABH3 cDNAs encode proteins of 119 amino acids, and combining these data with the N-terminal sequence we calculate that the mature ABH3 hydrophobins, starting with threonine, contain 94 amino acids [molecular mass = 9228 Da (strain 39) and 9271 Da (strain 97)]. Mass spectrometry using ABH3 isolated from U1 medium gave a main peak of 9107. Considering the precision of the method, this value can be matched with that from ABH3-97 if it is assumed that this sequence starts with an aspartic acid residue (molecular mass 9114 Da). This would again argue in favour of a non-glycosylated protein, in agreement with the absence of staining for carbohydrates. ABH3 conforms to the typical cysteine distribution pattern for basidiomycete hydrophobins although a slight deviation is observed: between the first and the second cysteine residues eight amino acid residues are found instead of six. The ABH3 hydrophobin contains only four negatively charged amino acids with a calculated protein PI of 2.68.

When compared to other class I hydrophobins, the ABH3 hydrophobin seems to be more related to the SC3 (50% identity) and SC1 (51%) hydrophobins of *S. commune* than to ABH1 or ABH2 (37%), the fruiting-body-specific hydrophobins from *A. bisporus*. However, if we compare the hydropathy plots of these hydrophobins they all show a high degree of similarity (Fig. 4).

**Expression of ABH3**

The levels of ABH3 transcript were compared at two stages of development of the heterokaryotic strain U1. Total RNA was extracted from colonies grown on compost agar (containing vegetative mycelium only) and from 3-d-old fruiting bodies grown on compost. DNA–RNA hybridizations were performed using the ABH1 and ABH3 cDNAs as probes. ABH3 transcripts were found in vegetative mycelium but not in fruiting bodies (Fig. 5a) while the ABH1 transcripts showed the opposite distribution pattern (Fig. 5b).

Homokaryotic parental strains H97 and H39 showed...
accumulation of the ABH3 transcript (Fig. 5c) but no ABH1 transcript was found in the homokaryons.

Colonies of the heterokaryon grown on compost medium showed a higher level of ABH3 transcripts than colonies grown on MMP medium. Colonies were first grown for 5 d on MMP medium on top of a perforated polycarbonate membrane and were then transferred to plates with either MMP or compost medium. After 2 d, colonies on MMP had very short brown-pigmented aerial hyphae while colonies on compost medium had developed longer whitish aerial hyphae. Total RNA was isolated from these colonies and hybridized to an ABH3 probe. After correction for differences in the amount of RNA applied, the ABH3 transcript level in the colony grown on compost medium was found to be eight times higher than that from the colony grown on MMP medium (Fig. 5d).

**Immunolocalization of ABH3**

At the colony level, secretion of ABH3 into the medium could be shown mainly at the expanding edge of the colony (Fig. 6a).

Mycelium grown on bare plastic showed labelling at the surface of the hyphae but particularly on the plastic surface where hyphae were detached during handling. When *A. bisporus* was grown in this way, small water droplets of about 10 μm diameter could be seen along the hyphae. Some of them apparently contained ABH3, visible after immunostaining (Fig. 6b). These experiments suggest that ABH3 causes adherence of *A. bisporus* hyphae to the hydrophobic plastic, similar to the role of SC3 in adherence of *S. commune* hyphae to hydrophobic substrates (Wosten et al., 1994a).

**Interfacial assembly of ABH3**

Discs of Teflon were immersed in an aqueous solution of ABH3 (approx. 20 μg ml⁻¹) for 16 h. The Teflon discs were then washed with water, dried, and contact angles of 1 μl water droplets were measured. Contact angle values dropped from 110° (bare Teflon) to 53 ± 1° (coated Teflon). After extraction of the ABH3-coated discs with 1% SDS at 100 °C for 10 min, the contact angle values increased to 59 ± 5°. These discs were further shown to react with the ABH3 antiserum before and after extraction with SDS. These data show that ABH3 is a class I hydrophobin. For measuring contact angles at the hydrophobic side of an ABH3 film, paper strips were coated with ABH3. As with ABH1 (Lugones et al., 1996), the paper became hydrophobic in an area just beneath the evaporating front. Water contact angles of 117 ± 3° were measured after extraction with hot SDS. Mycelium mats covered with aerial mycelium were extracted with chloroform/methanol (2:1, v/v, at 65 °C; five times, 10 min) and water contact angles were measured. Values of 120 ± 5° were found.

The surface activity of ABH3 (at 100 μg ml⁻¹) was measured by means of the Axi-Symmetric Drop Shape Analysis by Profile technique and a value of 37 mJ m⁻² was found which compares to 32 mJ m⁻² for SC3 of *S. commune*. This shows that ABH3 is also a highly surface-active protein.

To investigate the ultrastructure of assembled ABH3, a solution of the protein was allowed to dry on Formvar-coated grids and shadowed. The surface, representing...
the hydrophobic face, showed a mosaic pattern of parallel 10-nm-wide rodlets (Fig. 1b) similar to the structures found on mycelial cords (Fig. 1a).

**Phenotypic complementation of an SC3 mutant of S. commune by ABH3**

SC3 in the culture medium facilitates the breaching of the water–air surface by hyphae, probably by strongly reducing the surface tension (van der Vegt et al., 1996). Since ABH3 similarly reduces surface tension we investigated whether formation of aerial hyphae was restored by adding ABH3 or other hydrophobins to the culture medium of an S. commune strain, 72-3, in which the SC3 gene is disrupted (Wösten et al., 1994a; van Wetter et al., 1996). This strain forms few aerial hyphae, if formed at all (van Wetter et al., 1996), and formation of aerial hyphae can be restored by adding SC3 to the medium (H. A. B. Wösten, M.-A. van Wetter & J. G. H. Wessels, unpublished data). Homogenate of S. commune strain 72-3 mycelium (40 µl) was inoculated in small glass vials (width 14 mm, height 45 mm) containing 400 µl of minimal medium, with or without monomerized ABH3 or SC3. Abundant aerial hyphae were formed when either SC3 or ABH3 at 30–60 µg ml⁻¹ was added to the culture medium, in contrast to the culture not receiving a hydrophobin or receiving other proteins such as serum albumin (data not shown).

**DISCUSSION**

In this paper, we describe the isolation and characterization of a hydrophobin, ABH3, secreted by the substrate mycelium of heterokaryotic strain U1 of A. bisporus and by its parent homokaryons. On SDS-PAGE, ABH3 was found to run at a higher position than expected (19 kDa instead of 9 kDa). This has also been observed for other hydrophobins [SC3 and SC4 (Wessels et al., 1991) and ABH1 (Lugones et al., 1996)] and could reflect an anomalous binding of SDS by these proteins. The primary amino acid sequence of ABH3 was derived from the nucleotide sequence of cloned cDNA. It turned out that the heterokaryon produced two slightly different versions of ABH3, each encoded by one of the two different nuclei of the heterokaryon. Purified ABH3 exhibited the typical *in vitro* properties of a class I hydrophobin (Wessels, 1994) like those observed for the SC3 hydrophobin of S. commune (Wösten et al., 1994a; Wessels, 1997).

A rodlet layer consisting of SDS-insoluble assembled ABH3 was found to be present at the surface of aerial mycelium and on mycelial cords observed during cultivation of A. bisporus on compost medium. Both emergent structures grow into the gas phase and we assume that ABH3 secreted at the tips of growing hyphae self-assembles when it contacts the gas phase, as shown for the SC3 hydrophobin of S. commune (Wösten et al., 1993, 1994b). In addition, the tremendous lowering of the surface tension by assembled ABH3 may facilitate the breaching of the water–gas interface by hyphae. In S. commune, it has been shown that disruption of the SC3 gene greatly diminished the ability of the mycelium to form aerial hyphae (van Wetter et al., 1996) but formation of aerial hyphae, though of a hydrophilic nature, could be restored by adding SC3 to the medium. The fact that ABH3 could substitute for SC3 in this test suggests that ABH3 may fulfil a similar role in A. bisporus. Interestingly, the amino acid sequence of ABH3 is 50% identical to SC3 of S. commune, but shows only about 37% identity to the other hydrophobins of A. bisporus [ABH1 (HYPA) and ABH2 (HYPC); Lugones et al., 1996; de Groot et al., 1996].

Whereas the ABH1 and ABH2 genes were typically expressed in the fruiting bodies of the heterokaryon and not in the substrate mycelium of heterokaryon and homokaryon (Lugones et al., 1996; de Groot et al., 1996), ABH3 was specifically expressed in the substrate mycelium of both the heterokaryon U1 and the parent homokaryons of A. bisporus. This very much resembles the type of regulation of hydrophobin genes in S. commune, in which SC3 is expressed in both the primary and the secondary mycelium whereas SC1, SC4 and SC6 are expressed in the secondary mycelium only (Mulder & Wessels, 1986). Differential expression of these hydrophobin genes is clearly under control of the mating-type genes (Wessels, 1992; Wessels et al., 1995). The expression of SC3 in the secondary mycelium of S. commune has been attributed to the fact that in some hyphae, particularly in incipient aerial hyphae, the binucleate state is disrupted and nuclei with different mating-type genes are positioned at a considerable distance, lessening their interaction, resulting in expression of SC3 but precluding expression of the other hydrophobin genes (Asgeirsdóttir et al., 1995; Wessels et al., 1995). If such nuclear interactions also govern hydrophobin gene expression in A. bisporus, then any genetical or environmental condition that disturbs the normal juxtaposition of nuclei of opposite mating type could lead to diminished fruiting and excessive formation of aerial hyphae during commercial mushroom cultivation.

The coating of mycelial cords in the substrate mycelium of A. bisporus with ABH3 could play an important role during growth in compost, enabling the mycelium to switch from exploitative to explorative behaviour while crossing non-nutritive areas such as gas spaces (Rayner et al., 1991). However, the exploitation of the lignocellulose substrate could also be aided by secretion of ABH3. The abundant secretion of monomeric ABH3 would render a hydrophobic substrate like lignin hydrophilic and promote a close attachment between the assimilative hyphae and the lignin, as suggested by the observed wetting activity of ABH3 and the attachment of hyphae of A. bisporus to the hydrophobic plastic of a Petri dish. This adherence is probably caused by the formation of an amphipathic membrane of assembled ABH3 positioned between the hydrophilic wall and the hydrophobic plastic because an antibody clearly localized ABH3 on the plastic where a hypha had become detached. Apparently the hydrophobic interactions were
stronger than the hydrophilic interactions at the cell wall hydrophobin interface. In *S. commune*, such an attachment of hyphae to Teflon by SC3 was proven by localizing SC3 at the junction between the hypha and the plastic and by the diminished attachment after disruption of the SC3 gene (Wosten *et al.*, 1994a). Whereas *S. commune* is a poor lignin degrader (Schmidt & Liese, 1980), *A. bisporus* actively degrades lignin in the compost (Durrant *et al.*, 1991). ABH3 could thus play an important role in the degradation of lignin, causing tight adherence of hyphae to this substrate. A function of ABH3 in lignin degradation is made more plausible by the recent observation (H. A. B. Wosten & H. Engelhardt, unpublished data) that membranes formed by class I hydrophobins are not impermeable structures but contain pores that could allow the passage of small oxidizing molecules and the retrieval of lignin degradation products.

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