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HYDROPHOBINS: Multipurpose Proteins

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■ **Abstract** Class I and class II hydrophobins are small secreted fungal proteins that play a role in a broad range of processes in the growth and development of filamentous fungi. For instance, they are involved in the formation of aerial structures and in the attachment of hyphae to hydrophobic surfaces. The mechanisms by which hydrophobins fulfill these functions are based on their property to self-assemble at hydrophilic-hydrophobic interfaces into a 10 nm-thin highly amphipathic film. Complementation studies have shown that class I hydrophobins belong to a closely related group of morphogenetic proteins, but that they have evolved to function at specific interfaces. Recent evidence indicates that hydrophobins do not only function by self-assembly. Monomeric hydrophobin has been implicated in cell-wall assembly, but the underlying mechanism is not yet clear. In addition, hydrophobin monomers could act as toxins and elicitors.

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INTRODUCTION

Filamentous fungi colonize living or dead moist substrates by means of hyphae that extend at their apices while branching subapically. After a submerged feeding mycelium has been established, aerial hyphae are formed, which may develop into reproductive structures such as conidiophores or fruiting bodies (e.g. mushrooms or brackets). Spores that are formed by these structures are dispersed and may give rise to new colonizing mycelia. In these and other stages in the life cycle of filamentous fungi, small secreted proteins, called hydrophobins, fulfill a broad spectrum of functions (88, 98).

Hydrophobins are proteins that occur uniquely in mycelial fungi. These proteins and their encoding genes have been isolated from ascomycetes and basidiomycetes (see 88; Figure 1). Some evidence indicates that hydrophobins occur in zygomycetes as well (24), but it is not yet clear whether they occur in the chytridiomycetes.

Figure 1 Comparison of the deduced amino acid sequences of the identified class I and class II hydrophobin genes *SC3* of *Schizophyllum commune* (23), *ABH3* of *Agaricus bisporus* (43), *POH3* of *Pleurotus ostreatus* (1), *POH2* of *P. ostreatus* (1), *COH1* of *Coprinus cinereus* (2), *VMH1* of *P. ostreatus* (37), *COH2* of *C. cinereus* (2), *Hyd2* of *Lentinula edodes* (49), *SC1* of *S. commune* (60), *SC6* of *S. commune* (90), *HypB* of *A. bisporus* (18), *SC4* of *S. commune* (60), *Aa-Pri2* of *Agrocybe aegerita* (57), *FBH1* of *P. ostreatus* (51), *POH1* of *P. ostreatus* (1), *ABH1/HYPA* of *A. bisporus* (19, 41), *ABH2/HYPC* of *A. bisporus* (19, 41), *Hyd-Pt1* of *Pisolithus tinctorius* (72), *Hyd-Pt2* of *P. tinctorius* (72), *Hyd-Pt3* of *P. tinctorius* (D. Tagu, unpublished data; AAC95356), *VMH3* of *P. ostreatus* (37), *Hyd1* of *L. edodes* (49), *Hum2* of *U. maydis* (7), *MPG1* of *Magnaporthe grisea* (76), *RodA* of *Aspergillus nidulans* (69), *Hyp1* of *Aspergillus fumigatus* (50, 80), *DewA* of *A. nidulans* (70), *SsgA* of *Metarhizium anisopliae* (68), *XEH1* of *Xanthoria ectaneoides* (58), *XPH1* of *Xanthoria parietina* (58), *Hcf1* of *Cladosporium fulvum* (66), *Hcf2* of *C. fulvum* (62), *Hcf3* of *C. fulvum* (62), *Hcf4* of *C. fulvum* (62), *Eas* of *Neurospora crassa* (6, 38), *Tri1, Tri2, Tri3* of *the CFTH1* gene of *Claviceps fusiformis* (25), *Qid3* of *Trichoderma harzianum* (40), *Cry* of *Cryphonectria parasitica* (102), *Mag* of *M. grisea* (S.-O. Kim et al., unpublished data, AAD18059), *Hfb1* of *Trichoderma reesei* (48), *Hfb2* of *T. reesei* (47), *SrH1* of *T. harzianum* (46), *Cu* of *Ophiostoma novo-ulmin* and *O. ulmin* (10), *Hcf5* of *C. fulvum* (62), *Hcf6* of *C. fulvum* (P. Spanu, unpublished data). The alignment was done with the multalin program (17) using the dayhoff-8-0 settings and starting at the first cysteine residue of the hydrophobin because of the diversity in length and composition of the sequences preceding this residue. The eight conserved cysteine residues were used as fixed coordinates. Residues that are conserved in more than 50% of the hydrophobins are indicated in small font, while caps represent residues that are conserved in more than 90% of the cases. Note that *Tri1*, *Tri2*, and *Tri3* are contained in a single gene encoding the trihydrophobin of *C. fusiformis* (25).

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CLASS 1 HYDROPHOBINS FROM BASIDIOMYCETES
 SC3          CTT..GSLSC CNQ..VQSAS SSPV..... TALLGL.LGI V.L...SDLN VLVGI...SC S..PLTVIG. .VG.GSGCSA QTVCCENTQF NG.LINIGCT PINI.L
ABH3          CTAVGGDVNC CNS..LQEAS NPIV..... GLLAGL.LGI V.L...GPIQ GLVGL...TC N..PISIIG. ..G.GNSCSS QTVCCTGNNF SGGLLVIGCS PINIDL
POH3          CTT..GSLQC CNS..VQAAS NPVV..... GLLAGL.LGI V.L...GPIT GQVGL...TC S..PITVIG. .VG.GTSCSA QTVCCNGNSF NG.LIVVGCS PVNISL
POH2          CKT..GPVQC CNS..VQSSK SPAA..... SLLLGL.LGI V.L...QGVA VPVGL...TC N..PITVIG. .VG.GNSCSA QTVCCENNNF SG.LIAIGCT PINLSL
COH1          CNT..GPIQC CNT..VTQAS NPVA..... GLLLGL.LGI V.L...QDLN VLVGL...TC S..PISIIG. .LP.GNSCNA QPVCCQNNNF NG.LIAIGCT PININL
VMH1          CST..GTLNC CNS..SGTVE DKTI..... AGLLGI.LNI V.V...SDIT ALVGI..TC T..PITVVG. .AG.GTSCTS QTLCCDNNNF SG.LITLGCI PININL
COH2          CNAP.NNLEC CNS..VQAPT SGLI..... GTLLGL.LNI S.V...GDIT GLVGL...TC N..PISLIG. ..G.GNSCNA QTVCCQNNYF GG.LISIGCT PIIIDV
HYD2          CST..GDLQC CNT..VEPAS SPSA..... STILGL.LGI V.I...QGVD VLVGL...TC S..PITVIG. .LE.SGGCSA QAVCCTDNSN GG.LISIGCL PVTL..
SC1           CNS..GPVQC CNT.LVDTKD KHQT..... NIVGAL.LGL D.L...GSLT GLAGV...NC S..PVSVIG. .VG.GNSCST QTVCCEGTQF NG.LVNVGCT PINVGL
SC6           CNS..GTLQC CES.TTEAKD IDRV..... .LLSTL.LGV D.V...GSIT GLIGK...NC S..PVSVVG. .VGAGSTCST QTVCCDGDSF DG.LINLGCK SGNVAV
HYPB          CST..GPIEC CKQ.VMDSKS PQAT..... ELLTKNGLGL GVL...AGVK GLVGA...NC S..PITAIG. .IGSGSQCSG QTVCCQNNNF NG.VVAIGCT PINANV
SC4           CNS..GPVQC CNE.TTTVAN AQKQ..... GLLGGL.LGV V.V...GPIT GLVGL...NC S..PISVVG. .VLTGNSCTA QTVCCDHVTQ NG.LVNVGCT PISL..
AA-pri2       CNN..GSLQC CNSSMTQDRG NLQIAQGVLG GLLGGL.LGL GGLLDLVDLN ALIGV...QC S..PISIVG. ...NANTCTQ QTVCCSNNNF NG.LIALGCT PININL
FBH1          CGS..GSIQC CES..VQSAS AAQAA..... GILGP..LDI L.....TNLQ GLVGS...HC S..PLAAVG. .VS.GTSCSS QTVCCKDVSK SG.LVNLGCS PINLNL
POH1          CNT..GPIQC CNS..VQSAT SSAAA..... GPLAA..LGV L.....SGIA SLLGEVGLDC S..PLQVIG. .VG.ANSCSS QAACCTGNTF NG.AVVLGCS PIKL.L
ABH1/HYPA     CDV..GEIHC CDT...QQTP DHTS..... AAASGL.LGV P.....INLG AFLGF...DC T..PISVLG. .VG.GNNCAA QPVCCTGNQF TALINALDCS PVNVNL
ABH2/HYPC     CNV..GELHC CNT...QQTP DHTN..... AAG.GL.LGA A.....ANVG ALLGF...DC T..PISVIG. .IG.GNNCAA QPVCCEANEF TGLINALSCS PINVNL
HYD-Pt1       CNT..GPIQC CNT..VATSG S.QSG....V DELLTL.LGL S.....VPVG TQVGA...SC S..PISAVG. .TGSGAQCSG QTVCCEQNEW NG.LVNIGCM PINLNA
HYD-Pt2       CNT..GTPQC CQQ..VQQTS DLQ.Q....F RSSFGL.VDA L.....AGAS ALVGA...NC N..PVSVLG. .TGNGAQCNT QPVCCTSNQM LG.AVNMGCM PLNVNA
HYD-Pt3       CNT..GTIQC CQQ..VQQAS YYQSA....F QE.IGL.GEL L.....AGVT GQIGT...QC S..PISVVG. .ASNGAQCNA QTVCCTNTQF NG.LINIGCM PINVNA
VMH3          CNT..ESVKC CNK..SEDAE TFKK..... SASAAL.IPI K.I...GDIT GKVYS...EC S..PI..VG. .LIGGSSCSA QTVCCDNAKF NG.LVNIGCT PINVAL
HYD1          CST..GDQKC CQS..VQNSS AAGV..... SSLLGL.LGI V.L...SGTD VPVGL...TC L..PI..VG. ....GACQS QAVCCTDNSY GN.LISLGCS PLQL..
HUM2          CAV..GQAQC CSQ..VKQGK DAS..... DAIAGLGLAF N.....QILD GAIGL...DC QQIPVGVLGG AIAIQNTCKN TAVCCQGSAN NG.LIQTSCT PLSIN.
CONS          Cnt..G..qC Cn...vq... .......... ..l.gl.lg. .......... .lvG.....C s..Pisv.G. ..g.g..Cs. QtVCC..n.f nG.li..gC. Pin..l

CLASS I HYDROPHOBINS FROM ASCOMYCETES
MPG1          CGAEK.VVSC CNSKELKNSK S....GA... EIPIDVLSGE .......... .CKNIP.... INILTINQLI PINNFCSDTV S..CC..SGE QIG...LVNI Q..CTPI... LS.....
RODA          CGDQA.QLSC CNKATYAGDT TTVDEGLLSG AL.SGLIGAG SGAEGLGLFD QCSKLDV... AVLIGI..QD LVNQKCKQN. .IACCQNSPS SADGN.LIGV GLPCVALGSI L
HYP1          CGDQA.QLSC CNKATYAGDV TDIDEGILAG TL.KNLIGGG SGTEGLGLFN QCSNVDL..Q IPVIGIPIQA LVNQKCKQN. .IACCQNSPS DASGS.LIGL GLPCIALGSI L......
DEWA          C.NVG.SIAC C.....NSPA ETNNDSLLSG LLGAGLLNGL SGNTG....S ACAKASLIDQ LGLLALVDHT EEGPVCKNIV A..CCPEGTT N........ ...CVAVDNA GAGTKAE
SSGA          CDSG...VYC C.NKVAQNT. .....GI... VVPIDALSST .......... .C.GDT.... LKLVTVDALN DK...CTSQT V..CC.NNVQ QNG...LVNV A..CTPIDV. .......
XEH1          CSQGQ.TAKC C..NSLSKAV AN........ LIPIQV..GL N.....CVSLDL.... ISVLPIGKQ. .....CTQSQ ALACC.SSGQ QTG...LVNL GNVCVPVSL. .......
XPH1          CSQGQ.TAKC C..NSLSKAV AN........ LIPIQI..GL N.....CVSLDL.... ISVLPIGKQ. .....CTQSQ ALACC.SSGQ QTG...LVNL GNVCVPVSL. .......
HCF1          CAVGS.QISC CTTNSSGSD. .......... ILG.NVLGGS .......... .CLLDN.... VSLISS..LN SN...CPAGN TF.CCP..SN QDG...TLNI NVSCIPVSA. .......
HCF2          CAVGS.QVSC CTTDSSGSD. .......... VLG.NVLGGS .......... .CLVDN.... LSLISI..LN SQ...CPGAN TF.CCPSFTN QDG...TLNI HAACIPVAL. .......
HCF3          CATGA.QVAC CTTNSSNSD. .......... LLG.NVVGGS .......... .CLLDN.... LSLLSS..LN SN...CPAGN TF.CCP..SN SDG...TLNI NAQCIPISA. .......
HCF4          CAKGS.EISC CTTDSSNSG. .......... ALG.NVLGGS .......... .CLLQN.... LSLLSS..LN SN...CAAAN TF.CCP..TT QEG...TLNI NLSCIPISL. .......
EAS           CSIDDYKPYC C..QSMSGPA GS..PGLLN. LIPVDLSASL G.....CV..... VGVIG..... ..SQ.CGASV K..CCKDDVT NTGNSFLIIN AANC..VA.. .......
CONS          C..g.....C C...s..... .......... .l....l.g. .......... .C........ ........l. .....C.... ...CC..... q.g.....n. ...C.p.... .......

CLASS II HYDROPHOBINS FROM ASCOMYCETES
TRI1          CPAGL.YSNP QCCATDVLGV ADLDCKNPSS APMSGDNFKS IC.NAVGQQA KCCVLPVAGQ AVLCQD... ...
TRI3          CPSGL.YSVP QCCATDVLGV ADLDCGNPSR QPTDSSDFAS VC.AAKGQRA RCCVLPLLGQ AVLCTGA.. ...
TRI2          CPAGLLYSNP QCCSTGVLGV ADLDCKNPSS APTSGDDFQK IC.ANGGQQA QCCSIPVAGQ AVLCQPAIG GG.
QID3          CPAGL.YSNP QCCATDVLGL ADLDCAVPST TPHDGPNFQS ICVANGGKRA RCCVLPVLGL GVLCQNPVG TN.
CRY           CSSTL.YSEA QCCATDVLGV ADLDCETVPE TPTSASSFES IC.ATSGRDA KCCTIPLLGQ ALLCQDPVG L..
MAG           C.SGL.YGSA QCCATDILGL ANLDCGQPSD APVDADNFSE IC.AAIGQRA RCCVLPILDQ GILCNTPAG VTP
HFB1          CPPGL.FSNP QCCATQVLGL IGLDCKVPSQ NVYDGTDFRN VC.AKTGAQP LCCVAPVAGQ ALLCQTAVG A..
HFB2          CPTGL.FSNP LCCATNVLDL IGVDCKTPTI AVDTGAIFQA HC.ASKGSKP LCCVAPVADQ ALLCQKAIG TF.
SRH1          CPNGL.YSNP QCCGANVLGV AALDCHTPRV DVLTGPIFQA VCAAEGGKQP LCCVVPVAGQ DLLCEEAQG TF.
CU            C.TGLLQKSP QCCNTDILGV ANLDCHGPPS VPTSPSQFQA SCVRDGGRQA RCCTLSLLGL ALVCTDPVG I..
HCF5          CPA...LDTP LCCQADVLGV LDLTCEAPSD D.TSVSNFEA AC.ATTGLTA RCCTLPLLGE ALLCTTP.. ...
HCF6          CPA...NRVP QCCQLSVLGV ADVTCASPSS GLTSVSAFEA DC.ANDGTTA QCCLIPVLGL GLFCSNP.. ...
CONS          Cp.gl....p qCC.t.vLGv adldC..Ps. .pts...F.. .C.a..G..a .CCvlPvlgq allC....g ...
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Based on their hydropathy patterns and solubility characteristics, class I and class II hydrophobins were identified (86). Class I hydrophobins have been identified in both ascomycetes and basidiomycetes. Until now, class II hydrophobins have been found in ascomycetes only.

Hydrophobins allow fungi to escape their aqueous environment (98), confer hydrophobicity to fungal surfaces in contact with air (6, 38, 41, 42, 69, 77, 80, 84), and mediate attachment of hyphae to hydrophobic surfaces (78, 97), resulting in morphogenetic signals (77). The latter is important in initial steps of fungal pathogenesis where the fungus must attach to the hydrophobic surface of the host before penetration and infection can occur. Moreover, hydrophobins seem to function in cases of symbiosis between fungi and plants (ectomycorrhizae) (72) or algae and/or cyanobacteria (lichens) (29, 58). The mechanism underlying all these functions is based on the property of hydrophobins to self-assemble at a hydrophilic/hydrophobic interface into an amphipathic membrane (93, 95–97). Upon self-assembly at the interface between the hydrophilic cell wall and a hydrophobic environment (the air or the hydrophobic surface of a host), the hydrophilic side of the amphipathic membrane orients and attaches itself to the cell wall, while the hydrophobic side becomes exposed to the hydrophobic environment. Aerial hyphae and spores thus become hydrophobic, whereas hyphae that grow over a hydrophobic substrate attach themselves.

Recently, it was shown that hydrophobins not only function at hydrophilic-hydrophobic interfaces but also within the matrix of the cell wall where they influence cell wall composition (83). The mechanism is not yet clear. Moreover, the class II hydrophobin cerato-ulmin is a toxin for elm that seems to function as a monomer by increasing the permeability of the plant plasma membrane (67).

In this overview the role of hydrophobins in fungal development is described. Most of it is based on the properties and the functions of SC3 of *Schizophyllum commune*, which is the best studied hydrophobin.

INTERFACIAL SELF-ASSEMBLY OF HYDROPHOBINS

Class I and class II hydrophobins are about 100 amino acids in length, have characteristic hydropathy patterns, and contain eight conserved cysteine residues (86, 88) that form intramolecular disulphide bridges (24, 101). Hydrophobins may be glycosylated, but the properties of these proteins can be solely attributed to their amino acid sequences (94). Yet, although the amino acid sequences of class II hydrophobins are relatively well conserved, those of the class I hydrophobins show a low homology (Figure 1). It would be hard, if not impossible, to design universal primers to pick up class I hydrophobin genes by polychain reaction (PCR). In Figure 1, the sequences of identified class I and class II hydrophobins are given starting at the first cysteine residue. The sequences preceding this residue are of variable composition and length (from 26 to 158 and 17 to 73 amino acids, respectively) and include signal sequences of about 20 amino acids that are cleaved in the secretory pathway.

All hydrophobins that have been physically isolated self-assemble at hydrophilic-hydrophobic interfaces into amphipathic membranes (13, 41–43, 52, 53, 56, 74, 75, 93, 95–97). One side of the hydrophobin membrane is moderately to highly hydrophilic (water contact angles ranging between 22° and 63°), while the other side exposes a surface as hydrophobic as Teflon or paraffin (water contact angle 110°) (see 94). The membranes formed by class I hydrophobins (e.g. those of SC3 and SC4 of *S. commune*) are highly insoluble [resisting 2% sodium dodecyl sulphate (SDS) at 100°C] and can only be dissociated by agents such as formic acid (FA) or trifluoroacetic acid (TFA) (24, 91, 92). In contrast, membranes of the class II hydrophobins cerato-ulmin (CU) of *Ophiostoma ulmi* and cryparin (CRP) of *Cryphonectria parasitica* readily dissociate in 60% ethanol and in 2% SDS (13, 56), while assembled CU is also known to dissociate by applying pressure or by cooling (56).

Self-assembly of hydrophobins is accompanied by conformational changes. Monomeric class I and class II hydrophobins are rich in β-sheet structure (23, 101). At the water-air interface, class I hydrophobins attain more β-sheet structure (called the β-sheet state), while at the interface between water and a hydrophobic solid, a form with increased α-helix is observed (the α-helical state) (23). The α-helical state seems to be an intermediate of self-assembly, whereas the β-sheet state is the stable end-form. At the water-air interface, monomers of class I hydrophobins attain the α-helical state within seconds, but the conversion to the β-sheet state is much slower and takes minutes to hours (M.L. de Vocht, unpublished data). At the water-solid interface, the protein also readily attains the α-helical state but is thought to be arrested in this intermediate state. The β-sheet end state can only be reached by applying a combination of heat and diluted detergent (94). Both forms of the assembled hydrophobin have an amphipathic nature and can be dissociated with TFA, which unfolds the protein. After removing the solvent and dissolution in water, class I hydrophobins refold to the same monomeric structure that was observed before purification or TFA treatment (23), and the process of self-assembly can be repeated (95). It is not yet known which structural changes accompany self-assembly of class II hydrophobins. However, self-assembly and disassembly of class II hydrophobins can also be repeated even after dissociation of the membrane by TFA. This shows that both classes of hydrophobins are highly resilient to this type of treatment.

The membrane of class I hydrophobins is characterized by a mosaic of bundles of 5–12 nm-wide parallel rodlets (6, 38, 41–43, 69, 77, 95). In contrast, rodlets have not been found at surfaces of the assembled class II hydrophobins CFTH1 of *Claviceps fusiformis* (25), CRP of *C. parasitica* (94), and HFB1 and HFB2 of *Trichoderma reesei* (S. Askolin & H.A.B. Wösten, unpublished data). Whether the absence of rodlets or the differences in rodlet diameter has any functional significance is not yet known. The rodlets of the class I hydrophobins, SC3 and SC4, of *S. commune* are very similar to the fibrils formed by amyloid proteins. They consist of two tracks of 2–3 protofilaments with a diameter of about 2.5 nm each, have a high degree of β-sheet structure, and interact with the fluorescent dyes Thioflavine T (ThT) and Congo Red (23, 28, 34) (M.L. de Vocht & I. Reviakine, unpublished

data). In addition, SC3 and amyloid proteins self-assemble via intermediates and only above a critical concentration. It was suggested that amyloid fibril formation is common to many, if not all, polypeptide chains (14, 26, 36). However, because formation of amyloid fibrils is accompanied by loss of function or even disease (e.g. Alzheimer's disease) (34), evolution would have selected against the propensity to form such fibrils. Yet, one or two mutation(s) in a protein suffice to considerably increase the tendency to form amyloid fibrils (8). To our knowledge, hydrophobins are the first example of functional amyloids, with multiple functions in fungal development (see below). Recently, it was found that the four disulfide bridges of the SC3 hydrophobin are essential to prevent the protein from forming the amyloid structures in the absence of a hydrophilic-hydrophobic interface (22). When the disulphide bridges were reduced and the sulfhydryl groups blocked with iodoacetamide, the protein spontaneously assembled in water. Its structure was then indistinguishable from that of native SC3 assembled at the water-air interface. Apparently, the disulphide bridges of hydrophobins keep monomers soluble in water (e.g. within the cell or in the medium) and thus prevent precocious self-assembly. This would explain why all hydrophobins have eight conserved cysteine residues.

Hydrophobins belong to the most surface-active molecules. With a maximal lowering of the water surface tension from 72 to 24 mJ m^{-2} at 50 μg ml^{-1}, SC3 is the most surface-active protein known (98). Other hydrophobins are also highly surface active (25, 42, 43). Their surface-lowering activities are at least similar to those of traditional biosurfactants (for references see 33). In contrast to these surfactants, surface activity is not dependent on a lipid conjugate but is solely caused by the amino acid sequence. Moreover, while the maximal lowering of the surface tension by the traditional surfactants is attained within seconds, it takes minutes to hours in the case of class I hydrophobins. This is explained by the fact that hydrophobins lower the water surface only after self-assembly that is accompanied by conformational changes in the molecule (see above).

Despite the fact that hydrophobins have diverged considerably, their gross properties are similar. This flexibility is also illustrated by the fact that removing 25 out of 31 amino acids preceding the first cysteine residue of the SC3 hydrophobin by genetic engineering only affected the wettability of the hydrophilic side of the assembled hydrophobin (59). A most remarkable hydrophobin is the trihydrophobin CFTH1 of *C. fusiformis*. It contains three class II hydrophobin-like units, each preceded by a Gly-Asn-rich repeat (25) and still behaves like other class II hydrophobins.

THE ROLE OF HYDROPHOBINS IN ESCAPE OF HYPHAE FROM THE AQUEOUS ENVIRONMENT

The first step in formation of fungal aerial structures (including the elaborate fruiting bodies) is the escape of individual hyphae from the moist substrate into the air. The water-air interface, characterized by a high surface tension of 72 mJ m^{-2} is a barrier for aerial growth (98). This is based on the observation that initiation of

formation of aerial hyphae in *S. commune* correlated with a drop in water surface tension from 72 to 45 mJ m^{-2} and that most aerial hyphae formed at a medium surface tension of 30 mJ m^{-2}. The course of the drop in surface tension correlated with the production of the SC3 hydrophobin and can be explained by the ability of SC3 to reduce the surface tension as low as 24 mJ m^{-2} upon assembly at the water-air interface. A strain of *S. commune*, in which the *SC3* gene was disrupted (Δ*SC3* strain), produced few aerial hyphae and the surface tension of the culture medium was reduced to 45 mJ m^{-2} only (due to secretion of other molecules that have some surface activity). By adding SC3 to the culture medium of the Δ*SC3* strain, surface tension decreased to wild-type levels and formation of aerial hyphae was restored (98). Both the wild-type and the Δ*SC3* strain formed the first aerial hyphae at 45 mJ m^{-2}. It was concluded that this is the critical surface tension that hyphae of *S. commune* can overcome in order to grow into the air.

Other fungi also secrete highly surface-active class I hydrophobins into their culture medium (1, 43, 94), suggesting that lowering of the water surface tension by secretion of hydrophobins is a general condition that initiates aerial growth. Two of these hydrophobins, SC4 of *S. commune* and ABH3 of *Agaricus bisporus*, could substitute for SC3 in effecting aerial growth in a Δ*SC3* mutant of *S. commune* (43, 98). As mentioned, class II hydrophobins are also highly surface active, but it has not yet been established whether they are also instrumental in the escape of hyphae by lowering the water surface tension. The finding that formation of aerial hyphae in *O. novo-ulmi* and *O. ulmi* correlated with the formation of the class II hydrophobin CU (11, 78) may hint in this direction.

If lowering of the water surface tension was instrumental in formation of aerial hyphae, one would expect that any surface-active molecule would effect formation of aerial hyphae. However, many surface-active molecules are toxic because they interact with cellular membranes. In contrast to most surfactants, surface activity of hydrophobins is attained by the conformational change that occurs when monomers assemble (see above). The large complex of assembled hydrophobins is not expected to diffuse through the cell wall and to interact with the plasma membrane, explaining why these molecules are not toxic. Indeed, apart from the class I hydrophobins, ABH3 and SC4, only the small octapeptide streptofactin of the filamentous bacterium *Streptomyces tendae* (54) induces aerial growth in the Δ*SC3* strain of *S. commune* (98). This molecule is involved in escape of filaments of *S. tendae* from the aqueous environment (54) and was also proposed to self-assemble. It is thus the functional equivalent of SC3 in this bacterium. Interestingly, the fungal hydrophobin SC3 could complement a strain of *S. tendae* defective in streptofactin production and a strain of *Streptomyces coelicolor* defective in the production of the 18 aa peptide SapB, which is the equivalent of streptofactin in *S. coelicolor* (54, 81). Apparently, filamentous bacteria and filamentous fungi evolved similar mechanisms to grow into the air to form reproductive structures, but the effective molecules have different structures.

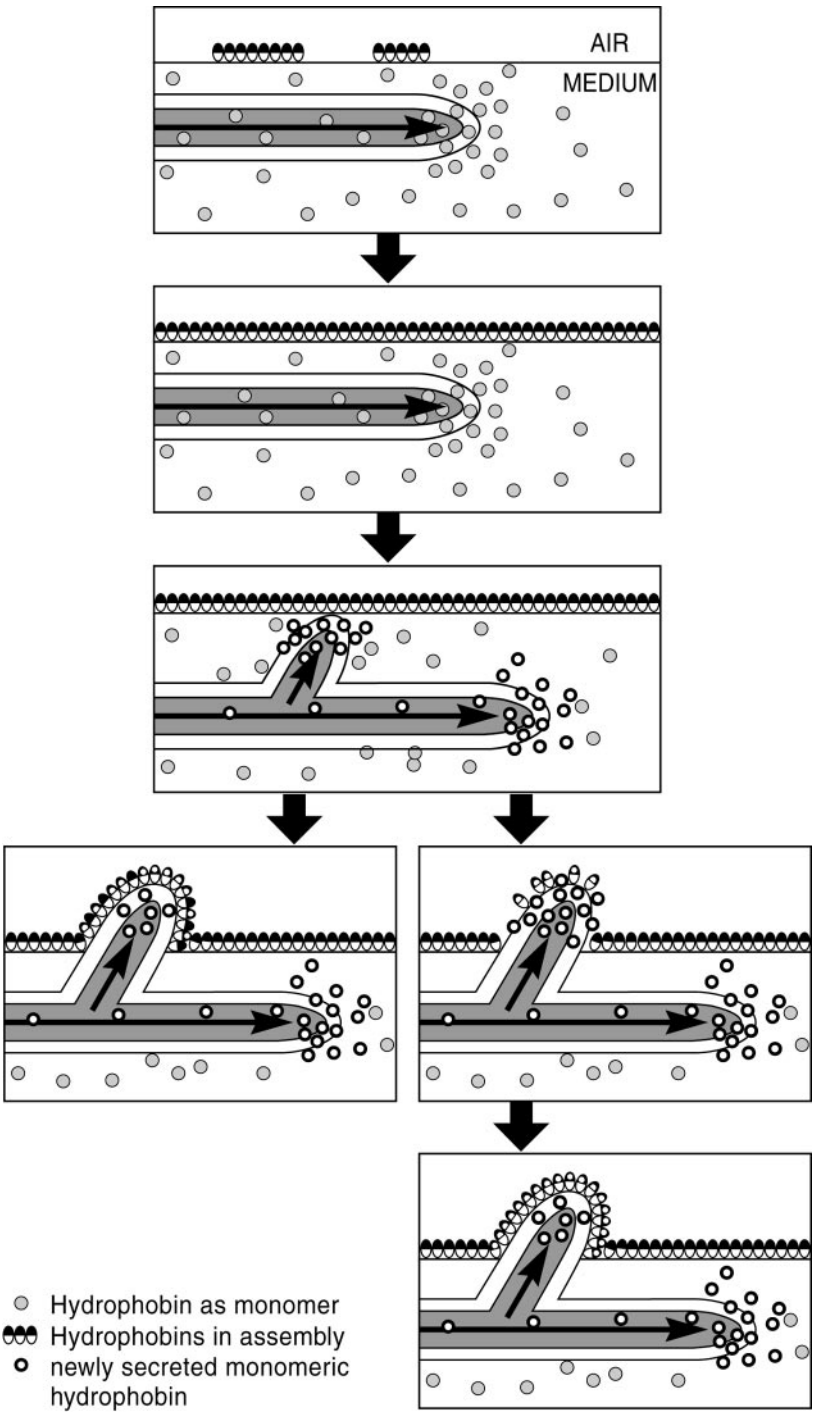
The membrane of the SC3 hydrophobin can be seen at the water surface as a light-reflecting layer and can be picked up as a discrete film with a holey grid (W. Bergsma-Schutter and M.L. de Vocht, unpublished data). What happens

when a hypha of *S. commune* approaches the water surface that is covered with a hydrophobin membrane? It could be that a stretch in the hydrophobin film due to the force exerted by the turgor pressure of a hypha in contact with the membrane would allow intercalation of newly secreted SC3 monomers, thereby expanding the membrane without physicially breaking it (Figure 2). As a consequence, the aerial hypha would never leave the water, but the aqueous environment would be extended and covered with a continuous layer of hydrophobin. Alternatively, the hypha may puncture the hydrophobin membrane and its cell wall would make contact with the air. Hydrophobins secreted by such aerial hyphae would self-assemble at the cell wall–air interface (see below); this film may fuse with that at the medium interface, resulting in a continuous membrane (Figure 2). It is not yet known which model gives the best description for wild-type hyphae, but they do have the capacity to puncture the hydrophobin membrane. This is concluded from the fact that aerial hyphae that are formed by the Δ*SC3* strain of *S. commune* in a medium with exogenously added hydrophobin are hydrophilic and lack a hydrophobin membrane (98).

That surface tension of water could be a barrier for aerial growth came to us as a surprise. It was already shown in 1895 (45) that fungal hyphae of *Botrytus cinerea* and *Penicillium glaucum* can puncture 24-karat gold foil, suggesting that breaching a water surface should not be a problem. However, one should realize that in the case of the solid surface, the hyphae are firmly attached, which is essential to effectuate the mechanical force generated by the turgor pressure that drives the penetration. Hyphae in water are not attached and follow the way of least resistance and are thus easily deflected.

Aerial structures are fed by transport of water and nutrients from the submerged mycelium (88). As a consequence, a certain mass of feeding mycelium has to be established before aerial growth ensues. By repressing the expression of hydrophobin genes in juvenile mycelia, thereby maintaining the high water-surface tension of

Figure 2 Model for the formation of fungal aerial structures. After a submerged feeding mycelium has been formed, the fungus secretes monomeric hydrophobin into the medium. The hydrophobin monomers self-assemble at the medium-air interface into an amphipathic membrane, which is accompanied by a huge decrease in water surface tension. It has not yet been established what happens when a hypha approaches the water-air interface and is confronted with the amphipathic protein film. The force generated by the turgor pressure of the approaching hypha may stretch the hydrophobin film, enabling intercalation of newly secreted hydrophobin monomers without rupturing the membrane (*bottom, left*). In this way the hypha would in fact never leave the aqueous environment. Alternatively, the hydrophobin membrane is punctured by the hypha, and the cell wall contacts the air. Hydrophobin monomers secreted by such a hypha will self-assemble at the cell wall–air interface. The hydrophilic side of the hydrophobin film faces the hydrophilic cell wall, while its hydrophobic side is exposed to the air. The hydrophobin films covering the hyphae and the aqueous environment may fuse (*bottom, right*).



the environment, the fungus prevents precocious formation of aerial structures and is forced to colonize the aqueous substrate first. The onset of the *SC3* gene in *S. commune* is thus a regulatory switch for aerial growth. How the mass of the feeding mycelium is sensed and what signal is ultimately transduced to commence *SC3* expression are not known (100).

HYDROPHOBINS COAT AIR-EXPOSED FUNGAL SURFACES

Hydrophobins secreted by a hypha that has escaped its aqueous environment cannot diffuse into the medium but will be confronted with the cell wall–air interface. This induces formation of the amphipathic hydrophobin film, as was shown for SC3 of *S. commune* (93). The hydrophilic side of the protein film faces the hydrophilic cell wall, while its hydrophobic side, characterized by a rodlet pattern, is exposed (95). This confers hydrophobicity to the hypha.

Rodlet layers have generally been observed at surfaces of fungal aerial structures (e.g. conidiospores, conidiophores, and fruiting bodies) (see 87, 88). These layers are probably the result of assembly of class I hydrophobins in a way similar to that of SC3. Inactivation of class I hydrophobin genes of *Aspergillus nidulans*, *A. fumigatus*, *Neurospora crassa*, and *Magnaporthe grisea* (6, 38, 69, 77, 80) resulted in wettable conidiospores that lacked the rodlet layer. Class II hydrophobins also coat aerial structures. CRP was localized at surfaces of aerial hyphae, pycnidia (asexual spore-containing bodies), and fruiting bodies of *C. parasitica* (13), while CU was localized on surfaces of both submerged and emerged structures formed by aggressive isolates of *O. ulmi* (71). In addition, the HFB2 hydrophobin of *T. reesei* was isolated from fungal spores (47). Whether the lower stability of the assembled class II hydrophobin membrane has any effect on its role in aerial growth is unknown. It could be that the assembled membrane is stabilized by the interaction of its hydrophilic side with the cell wall possibly by a lectin-like interaction (13, see also below).

Fruiting bodies are the result of the interweaving of specialized aerial hyphae. Spaces between these hyphae are filled with a hydrophilic mucilage. The plectenchym tissue is traversed by an elaborate system of air channels, which probably serves gas exchange. The outer surface of fruiting bodies of *A. bisporus* and the walls of the air channels within these fruiting bodies were lined with the class I hydrophobin ABH1 (41, 42), while SC3 and SC4, respectively, coat these surfaces in *S. commune* (3, 42). The hydrophobin coating confers hydrophobicity, while the proposed porosity of the class I hydrophobin membrane would allow gas exchange (93). Formation of air channels was not affected in a Δ*SC4* strain of *S. commune*, but their walls were hydrophilic and, in contrast to those in wild-type fruiting bodies, easily filled with water (84). Indeed, the capillary force of air channels of *S. commune* is negative but would exceed one meter in the absence of the SC4 coating (note that the fruiting bodies are only a few centimeters in length). In the absence of the hydrophobin, gas exchange would thus easily become impaired under wet conditions.

Lichens are fungi associated with algae and/or cyanobacteria, which have reached the most ultimate form of aerial growth because, in contrast to other fungi, they always live above the substrate (88). The fungal hyphae make contact with the alga, together being ensheathed by a matrix that is covered with a rodlet layer at sites in contact with the air (29). Class I hydrophobins identified in *Xanthoria parietina* and *X. etaneoides* (58) may be responsible for this hydrophobic rodlet layer. As in air channels in fruiting bodies, the hydrophobin film would permit optimal gas exchange by preventing the air channels from becoming soaked with water during cycles of wetting and drying to which lichens are regularly subjected (29, 30, 87, 88).

Assembled hydrophobins are extremely resistant toward chemical and enzymatic treatment (see 93), and they may not only confer hydrophobicity but also protect emergent structures against adverse environmental conditions. It was suggested that they protect against desiccation (5, 95). Indeed, over-expression of CU in *O. ulmi* resulted in decreased susceptibility to desiccation, whereas susceptibility was increased in strains of *O. novo-ulmi* in which the *cu* gene was disrupted (78). However, differences in evaporation of water were not observed in an in vitro system in the presence or absence of an assembled surface layer of the class I hydrophobin SC3 (H.A.B. Wösten & S. Heys, unpublished data), which agrees with the noted porosity of the SC3 membrane (93). Possibly class I and class II hydrophobins differ in this respect.

The ABH1 (HypA) membrane covering fruiting bodies of *A. bisporus* (41) was proposed to protect the mushroom against bacterial infection (19). A similar role was proposed for the HYPB hydrophobin of *A. bisporus* that seems to be located at the border of the cap and stipe tissue (18). The rodlets found on conidia of *Coccidioides immitis* (16), which is the causal agent of valley fever (coccidioidomycosis), shield multiple antigenic molecules in the cell wall and thus may represent a defense of the pathogen against attack by the host cellular immune system (15, 93). Like animals, plants have developed mechanisms to detect and defend themselves against pathogenic fungi. Hydrophobins may mask β-glucan and chitin (79) main components of the fungal cell wall, derivatives of which exhibit a high elicitor activity (55, 63, 64).

THE ROLE OF HYDROPHOBINS IN PATHOGENICITY

Apart from the proposed function to protect against the host defense system (see above), hydrophobins appear to be involved in pathogenicity in two ways. They act as toxins and/or attach fungal structures to the host surface, either generating morphogenetic signals or not.

Hydrophobins in Attachment

Many condiospores are characterized by a rodlet-decorated surface of a class I hydrophobin (6, 38, 69, 77, 80), whereas yeast-like cells of *O. ulmi* and *O. novo-ulmi*

are covered with a layer of the class II hydrophobin CU (78). These hydrophobin layers not only serve dispersal of the infectious propagules by wind or insect vectors but also serve attachment to the host surface. An isolated rodlet preparation from conidia of *Beauveria bassiana* bound equally well to insect cuticles as intact conidia (9), while a correlation was found between production of CU in strains of *O. ulmi* and *O. novo-ulmi* and attachment of yeast-like cells to surfaces of the bark beetle vector *Scolytus multistriatus* (78).

Once the infectious propagule is attached, it may colonize the host, which may be mediated by the formation of an infection structure called the appressorium. To penetrate the host by mechanical force, this structure has to attach firmly. The class I hydrophobin, MPG1, of the rice pathogen *M. grisea* (76) was involved in formation and attachment of appressoria. Both processes were greatly reduced in a Δ*MPG1* strain (76, 77). The mechanism by which appressoria of *M. grisea* initially attach to the hydrophobic surface of the host is probably similar to that of hyphae of *S. commune* to hydrophobic Teflon. SC3 monomers secreted at tips of growing hyphae of *S. commune* self-assemble at the cell wall–Teflon interface. As a result, the SC3 membrane bridges the incompatible surfaces of the hydrophilic fungal cell wall and the hydrophobic solid, the amphipathic nature of the membrane allowing strong attachment (97). Hydrophobic interactions probably mediate the strong interaction between the hydrophobic side of the SC3 membrane and the hydrophobic solid, while a lectin-like activity may mediate the strong affinity of the hydrophilic side of the membrane with the cell wall (84). In the case of *M. grisea*, these interactions should withstand the force exerted by the penetrating infection hypha, which is driven by a turgor pressure of up to 80 atm that is generated in the appressorium (20, 31). Otherwise, the appressorium would be lifted off the surface.

Many pathogenic fungi only germinate in a humid environment, and a hydrophilic mucilage was implicated in attachment (27). However, hydrophobins may also be involved in this process (86). Hydrophobin secreted in the aqueous environment could self-assemble at the host surface, making it hydrophilic. This change in surface wettability may facilitate the interaction of the hydrophilic mucilage with the host surface. This hypothesis is supported by the observation that formation of appressoria on a hydrophobic surface by a Δ*MPG1* strain was restored by co-inoculation with a wild-type strain in an aqueous environment (4). Until now, only MPG1 has been shown to be instrumental in pathogenicity by attaching the fungus to the host surface. Yet, it is expected that this is a general mechanism. For instance, the hydrophobin gene *ssgA* is expressed during in vitro appressoria formation of the insect pathogen *Metarhizium anisopliae* (68). Whether a class II hydrophobin membrane could function in strong attachment is not yet known.

Interestingly, hydrophobin-mediated attachment appears to generate signals for further development. Addition of cAMP bypasses the need of *M. grisea* for an inductive hydrophobic substrate (39) and restores appressorium formation in the Δ*MPG1* strain (4, 77). Deformation of a hypha, resulting from strong attachment (97), might stretch the plasma membrane, activating mechano-sensitive channels

(86). This signal may be transduced via cAMP, ultimately leading to appressorium formation.

Hydrophobins as Toxins

The class II hydrophobin CU of *O. novo-ulmi* and *O. ulmi* had already been proposed to be a toxin in 1974 (73). This was based on the observation that injection of purified CU into the host white elm caused wilting, reduction in transpiration, increase in leaf respiration, and electrolyte loss. The toxicity of CU in in vitro experiments appeared to be host selective (52). It was suggested that the symptoms resulted from plugging xylem vesicles by CU-coated air bubbles (32, 56) and/or increased host plasma membrane permeability (67). However, pathogenicity was not affected when the *cu* gene was disrupted in *O. novo-ulmi* (11, 78), while overexpression of *cu* in the relatively nonaggressive *O. ulmi* did not increase pathogenicity (78). Moreover, two naturally occurring isolates of *O. novo-ulmi* that exhibited a flat morphology were deficient in CU production but were still pathogenic (12). On the other hand, when the *cu* gene of *O. novo-ulmi* was introduced in *O. quercus*, this fungus became pathogenic not only for weakened hardwood but for elm as well (21). Based on these data, it was proposed that CU is one of several pathogenicity factors in *O. novo-ulmi*, each being dispensable but together giving full pathogenicity (11). Many fungi produce more than one hydrophobin, and it may very well be that *O. novo-ulmi* and *O. ulmi* express other hydrophobins *in planta* as well (86). These hydrophobins could account for (a subset of) the pathogenicity factors.

If clogging the xylem causes wilting in *Ophiostoma*, then all hydrophobins would be potential toxins because they all can stabilize air bubbles by self-assembly. The solubility characteristics of class II hydrophobins (see above) would make them more effective to clog the xylem than the class I hydrophobins. Once a class II hydrophobin–coated air bubble collapses, the protein can be "recycled" to stabilize another air bubble. In contrast, in the case of a class I hydrophobin, a sheet of insoluble aggregated hydrophobin remains. Yet, no other hydrophobin acts as a toxin, although several hydrophobins have been isolated from pathogenic fungi. This and the host-specific effects of CU indicate a direct toxicity of hydrophobins (e.g., by interacting with the plasma membrane) rather than an indirect damage due to the physico-chemical properties of the molecule. It would be worthwhile to systematically test the ability of a variety of hydrophobins to increase pathogenicity of *Ophiostoma* species.

Apart from acting as toxins, hydrophobins could also act as elicitors (79, 86) to induce the plant defense system. However, until now no experimental evidence for this has been reported.

HYDROPHOBINS AFFECT HYPHAL WALL COMPOSITION

The cell wall of filamentous ascomycetes and basidiomycetes consists mainly of a complex of (1-3)/(1-6)β-glucan, chitin, (1-3)β-glucan, and (glyco)-proteins. The individual cell wall molecules are extruded into the wall at the growing hyphal

tip (85, 86). Within the wall the precursors of the glucan-chitin complex, water-soluble (1-3)β-glucan and chitin, are cross-linked, and the glucan is modified by introduction of (1-6)β linkages. The resulting complex rigidifies the cell wall. Apart from the insoluble (1-3)/(1-6)β-glucan linked to chitin, many filamentous fungi produce a water-soluble (1-3)β-glucan with single (1-6)β-linked glucose residues attached (called slime or mucilage), similar in structure to the glucan linked to chitin. The mucilage occurs freely in the wall and in the medium, and it is not yet known whether it is a precursor or degradation product of the glucan-chitin complex (65).

Unexpectedly, hydrophobins were shown to affect the cell wall composition (83). A young wild-type culture not yet expressing the *SC3* gene produced more water-soluble mucilage than a culture expressing the hydrophobin gene while the amount of glucan linked to chitin was lower. The composition of the cell wall of a Δ*SC3* strain did not change throughout culturing and was similar to that of young wild-type cultures. The Δ*SC3* strain could be complemented by reintroduction of the *SC3* gene. Complementation was also obtained using the *SC3* regulatory sequences and the coding sequences of the hydrophobin genes *SC4* of *S. commune* or *ABH1* of *A. bisporus*.

The mechanism by which SC3 influences the cell wall is not yet clear, but it seems to function within the matrix of the cell wall, implying that self-assembly is not involved. Hardly any SC3 was isolated from cell walls of submerged wild-type mycelium (83), indicating that SC3 does not fulfill a structural role in cell wall assembly. However, for a function in cell wall biosynthesis SC3 need not necessarily be retained in the cell wall. SC3 could fulfill its role during its transit through the cell wall. Once SC3 has reached the hyphal surface, it could diffuse into the medium. Recently, it was shown that monomers of SC3 can interact with the mucilage (44), suggesting that they could also interact with the glucan precursors of the glucan-chitin complex, possibly via a lectin-like activity (84).

It is not yet known what role a change in cell wall composition upon expression of hydrophobins has in the fungal life cycle. As mentioned, the *SC3* gene is not expressed in young cultures but is switched on at the moment aerial structures are to be formed. Possibly, the changed cell wall composition makes the cell wall more rigid, which may be instrumental when the turgor is too low to keep aerial structures erect.

ARE HYDROPHOBINS TAILORED TO FULFILL SPECIFIC FUNCTIONS?

Several fungi contain more than one hydrophobin gene. Three hydrophobin genes have been identified in *Pleurotus ostreatus* (1), four in *S. commune* (60, 90) and *A. bisporus* (18, 19, 41, 43). Six hydrophobin genes have been identified in *Cladosporium fulvum* (62, 66; P. Spanu, unpublished data]. Isolation of hydrophobin genes

by cross hybridization is hampered by their low homology at the nucleotide level (see 88). Therefore, the number of these genes may even be higher. Why should a fungus produce more than one hydrophobin rather than using a generic protein? It has been suggested that this enables the fungus to express hydrophobins at different stages of development (35), or that this reflects different functions for different hydrophobins (84, 93). Yet, a specific hydrophobin gene can be expressed at different stages of development and the encoding protein can fulfill several functions. For instance, SC3 is involved in escape of hyphae into the air (98), in making aerial hyphae hydrophobic (82, 95), in attaching hyphae to hydrophobic surfaces (97), and in determining the cell wall composition (84). MPG1 coats conidiospores, is involved in elaboration of appressoria, and is required for full pathogenicity (76, 77). CU is involved in formation of aerial hyphae, is a fitness factor (78), and is a toxin for elm (21, 73). However, this does not mean that any hydrophobin can equally well perform the functions attributed to this class of proteins. To investigate this, seven class I hydrophobin genes of a variety of fungi playing distinct roles in conidiogenesis, fruiting body development, aerial hyphae formation, and infection structure elaboration were expressed in a Δ*MPG1* strain of *M. grisea* under regulation of the *MPG1* promoter (35). Of these, only the SC3 hydrophobin of *S. commune* failed to complement the MPG1 hydrophobin, at least partially (35). Functional complementation was also studied in *S. commune*. The Δ*SC3* strain of *S. commune* was provided with the *SC4* gene under regulation of the *SC3* promoter (84). Although *SC4* of *S. commune* normally lines air channels in fruiting bodies, it could substitute for *SC3* in formation of hydrophobic aerial hyphae. However, attachment of hyphae to hydrophobic surfaces was only partially restored. This was explained by the fact that the hydrophilic side of the SC4 membrane has a lower affinity for the cell wall of emergent hyphae of *S. commune* than SC3. This may be due to different lectin specificities of SC3 and SC4 (84), evolved for the interaction with different cell wall polymers exposed at surfaces of individual emergent hyphae and the extracellular matrix within fruiting bodies.

These complementation studies show that although class I hydrophobins can partially substitute for each other and are therefore a closely related group of morphogenetic proteins (35), they have evolved to fulfill specific functions. This is also indicated by a phylogenetic analysis. The class I hydrophobins SC3 of *S. commune* (82), ABH3 of *A. bisporus* (43), COH1 of *Coprinus cinereus* (2), and POH1 of *P. ostreatus* (1) have all been shown or proposed to be involved in formation of aerial hyphae. These hydrophobins are more related to each other than SC3 and ABH3 are to the other hydrophobins of *S. commune* and *A. bisporus*, respectively. This suggests that functional similarity is reflected in the primary sequence of hydrophobins. As described above, *SC3* did not complement *MPG1*. It would be interesting to test whether the other hydrophobins in this "functional group" complement *MPG1*.

As mentioned earlier, not only class I but also class II hydrophobins are involved in formation of aerial hyphae. It is unknown whether class I and class II can functionally replace each other. *C. fulvum* contain genes belonging to both

hydrophobin classes (62), which is also the case for *M. grisea* (for references, see Figure 1). It would be interesting to establish whether individual hyphae co-express class I and class II hydrophobins, whether they form mixed membranes, and what the properties of these membranes would be. In the case of dikaryotic hyphae of *S. commune*, individual hyphae produce either SC3 or SC4, but not both (61).

CONCLUSIONS

Hydrophobins have been isolated from ascomycetes and basidiomycetes (88, 93), and they may occur in zygomycetes as well (24). This indicates that these proteins already existed at least 400 million years ago (89). Hydrophobins evolved to fulfill a broad spectrum of functions in fungal development. The roles of these proteins in formation of aerial structures and in attachment are now relatively well understood. They are based on the capacity of hydrophobins to self-assemble at hydrophilic-hydrophobic interfaces. However, monomeric hydrophobins may also play a role in fungal development. It seems that hydrophobin monomers within the matrix of the wall affect the cell wall composition, but their precise role has yet to be established. In addition, monomeric hydrophobins may act as toxins and elicitors.

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