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Hydrophobin Genes Involved in Formation of Aerial Hyphae and Fruit Bodies in Schizophyllum

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Fungi typically grow by apical extension of hyphae that penetrate moist substrates. After establishing a branched feeding mycelium, the hyphae differentiate and grow away from the substrate into the air where they form various structures such as aerial hyphae and mushrooms. In the basidiomycete species *Schizophyllum commune*, we previously identified a family of homologous genes that code for small cysteine-rich hydrophobic proteins. We now report that the encoded hydrophobins are excreted in abundance into the culture medium by submerged feeding hyphae but form highly insoluble complexes in the walls of emerging hyphae. The *Sc3* gene encodes a hydrophobin present in walls of aerial hyphae. The homologous *Sc1* and *Sc4* genes, which are regulated by the mating-type genes, encode hydrophobins present in walls of fruit body hyphae. The hydrophobins are probably instrumental in the emergence of these aerial structures.

INTRODUCTION

Fungi, except for the unicellular yeasts, grow by means of hyphae that only extend at their apices (Wessels, 1986). The hyphae regularly branch and establish a mycelium that colonizes dead or living substrata. After the mycelium has obtained a certain mass, hyphae at the substrate/air interface emerge into the air where they form a woolly aerial mat (aerial hyphae) or become involved in the differentiation of reproductive structures that aid in the dissemination of asexual or sexual spores. In the Basidiomycetes, the best known of these aerial structures are the fruit bodies or basidiomes. Usually these fruit bodies consist of heterokaryotic hyphae formed by the mating of two homokaryotic mycelia. In specialized cells (basidia) in the fruit bodies, the two haploid nuclei of the heterokaryon fuse to form a diploid nucleus that immediately undergoes meiosis and produces four haploid basidiospores. Among the Basidiomycetes that make fruit bodies, *Schizophyllum commune* (Raper, 1988) and *Coprinus cinereus* (Casselton and Economou, 1984) are genetically the best studied. In these species, the homokaryon is a monokaryon (one nucleus per cell) and the heterokaryon is a dikaryon (two genetically different haploid nuclei per cell). The dikaryon arises by mating two monokaryons with different alleles at two complex mating-type genes, *A* and *B*.

Several genes specifically activated at the time of fruit body formation in the dikaryon of Schizophyllum were cloned (Dons et al., 1984a; Mulder and Wessels, 1986). Among these, the genes *Sc1* and *Sc4* are noteworthy because their mRNAs are very abundant (Mulder and Wessels, 1986; Ruiters et al., 1988). Sequence analyses (Dons et al., 1984b; Schuren and Wessels, 1990) showed that these two genes, which are coordinately regulated by the mating-type genes (Ruiters et al., 1988) and the *FBF* gene (Springer and Wessels, 1989), are homologous and share extensive sequence homology with the *Sc3* gene that is very active in both monokaryons and dikaryons at the time that aerial hyphae are formed (Mulder and Wessels, 1986). The putative polypeptides encoded by the members of this gene family are quite hydrophobic with averaged hydrophobicity indices (Kyte and Doolittle, 1982) of +0.54, +0.59, and +0.90 for pSc1 (109 amino acids), pSc4 (111 amino acids), and pSc3 (125 amino acids), respectively. Another characteristic is that they all contain eight cysteines at conserved positions and putative signal sequences for secretion. We call these proteins "hydrophobins," a term coined for hydrophobic substances present in the walls of many prokaryotic and eukaryotic microorganisms (Doyle and Rosenberg, 1990).

In this study, we report that the hydrophobins encoded by the *Sc1/Sc4/Sc3* gene family of Schizophyllum are excreted into the medium by the substrate hyphae but form highly insoluble complexes in the walls of emerging hyphae where they appear to play important roles in morphogenesis. A mutation known as *thn* (Wessels et al., 1991) prevents accumulation of all hydrophobins and the emergence of all aerial structures.

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RESULTS

Expression of the Hydrophobin Gene Family

Figure 1 shows the morphological appearances of monokaryon and dikaryon, and of strains that carry mutations affecting the formation of aerial hyphae and fruit bodies, after 4 days of growth as a mycelial lawn. The fruiting heterokaryotic dikaryon *A41 B41/A43 B43* was synthesized by mating monokaryons with different mating-type genes (*A41 B41* and *A43 B43*). The homokaryotic mimic of the dikaryon (*Acon Bcon*) carries constitutive mutations in both mating-type genes (Raper, 1988). Both fruiting dikaryons make few aerial hyphae. The recessive mutation *thn* suppresses formation of aerial hyphae in monokaryons and suppresses formation of both aerial hyphae and fruit bodies in dikaryons (Schwalb and Miles, 1967; Wessels et al., 1991). The recessive mutation *fbf* has no phenotype in monokaryons but suppresses fruiting in dikaryons while allowing for abundant formation of aerial hyphae (Springer and Wessels, 1989).

Levels of mRNAs were determined in 4-day-old surface cultures of homokaryons and heterokaryons with or without the above-mentioned mutations (see Figure 1). Table 1 shows that the accumulation of mRNA from the *Sc1* and *Sc4* genes is controlled by the mating-type genes and by the *THN* and *FBF* genes and suggests that these mRNAs are involved in fruiting. Accumulation of mRNA from the *Sc3* gene appears to be controlled by the *THN* gene only, and its expression coincides with formation of aerial hyphae. It should be noted that after 2 days of growth all cultures looked much the same with no aerial hyphae and no fruit bodies, whereas the mRNAs for the hydrophobin genes were all at a very low level (Mulder and Wessels, 1986; data not shown).

Table 1. Abundance of Hydrophobin mRNAs in Various Strains of Schizophyllum Correlated with the Occurrence of Fruit Bodies and Aerial Hyphae

Genotype[a]	Fruit Bodies	Aerial Mycelium	mRNA Abundance[b]		
			Sc1	*Sc4*	*Sc3*
A41 B41	−	+	ND[c]	3.6	49.5
A43 B43	−	+	ND	5.1	63.5
A41 B41/B43 B43	+	+/−	30.6	98.4	41.4
Acon Bcon	+	+/−	34.2	96.2	36.6
Acon Bcon fbf	−	+	ND	2.7	84.0
A41 B41 thn	−	−	ND	ND	2.8
A43 B43 thn	−	−	ND	ND	ND
A41 B41 thn/A43 B43 thn	−	−	ND	ND	ND

[a] All strains were coisogenic except for the genes indicated and grown as a mycelial lawn for 4 days.
[b] mRNA abundance is given as percent (×1000) of total RNA. Maximum variation between mRNA values for replicate cultures was 5% of the values given.
[c] Not detected; abundance $<0.5 \times 10^{-3}$%.

Detection of the Proteins Encoded by the Hydrophobin Genes

To detect the putative hydrophobin proteins in vivo, the strains shown in Figure 1 were grown in the presence of $^{35}SO_4^{2-}$. All cultures incorporated the label at about the same rate. Analysis of hot-SDS-extracted proteins from whole mycelia by SDS-PAGE did not reveal significant differences between the strains (data not shown). Because of the putative presence of signal peptides (Schuren and Wessels, 1990), special attention was given to the possible presence of hydrophobins in cell walls and culture medium.

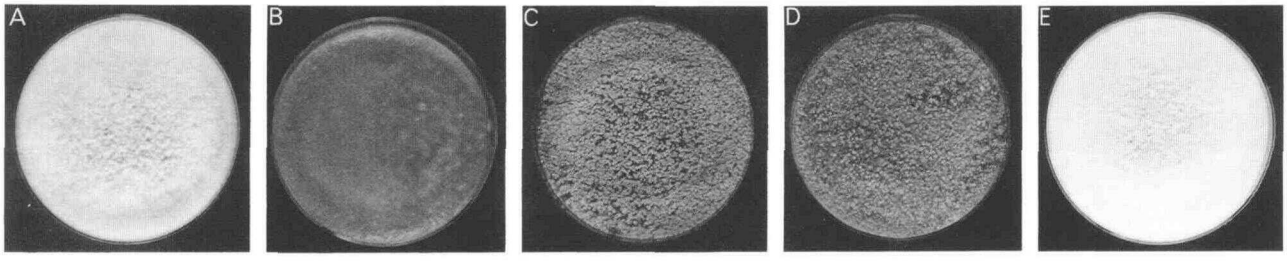

Figure 1. Morphological Appearance of 4-Day-Old Cultures of Coisogenic Schizophyllum Strains Variously Exhibiting the Ability to Produce Aerial Mycelium and Fruit Bodies.

(A) *A41 B41.*
(B) *A41 B41 thn.*
(C) *A41 B41/A43 B43.*
(D) *Acon Bcon.*
(E) *Acon Bcon fbf.*

Indeed, antibodies raised against synthetic peptides, based on unique sequences around the first intron of the *Sc1* and *Sc4* genes (Schuren and Wessels, 1990), specifically interacted with the cell walls and with medium proteins of strains expressing these genes but did not react with extracted proteins after SDS-PAGE (data not shown). Cell wall preparations of ^{35}S-labeled mycelia, therefore, were treated with hot SDS and the extracts were examined by SDS-PAGE. Figure 2A shows that no significant differences in protein patterns between the strains could be observed. However, it was noted that from walls expected to contain hydrophobins, only about half of the radioactivity was extracted by SDS. More than 80% of the remaining radioactive material could be extracted by cold formic acid. After removal of the formic acid, this material did not migrate in SDS-PAGE, indicating a high molecular weight. Because thiol agents like mercaptoethanol and dithiothreitol failed to dissociate these complexes, we oxidized all putative disulfide links between molecules with performic acid, converting cysteine and cystine to cysteic acid. Now the radioactive material migrated in SDS-PAGE, producing protein patterns (Figure 2B) consistent with the interpretation that the band at the 28 kD position represents pSc3 and the band at 19 kD represents pSc4, the two proteins expected to be most abundantly present. Examination of proteins precipitated from culture media of these labeled mycelia by SDS-PAGE revealed a complex pattern of bands (Figure 2C), but, after dissolution in formic acid and oxidation with performic acid, a pattern was obtained quite similar to that obtained from the cell walls (Figure 2D). The walls of the fruiting dikaryons contained very little 28-kD protein, which correlates with the near absence of aerial hyphae in these cultures. None of the heavily labeled protein bands visible in Figures 2B and 2D stained with Coomassie Brilliant Blue R 250. After blotting and staining, they appeared white against a faintly blue background.

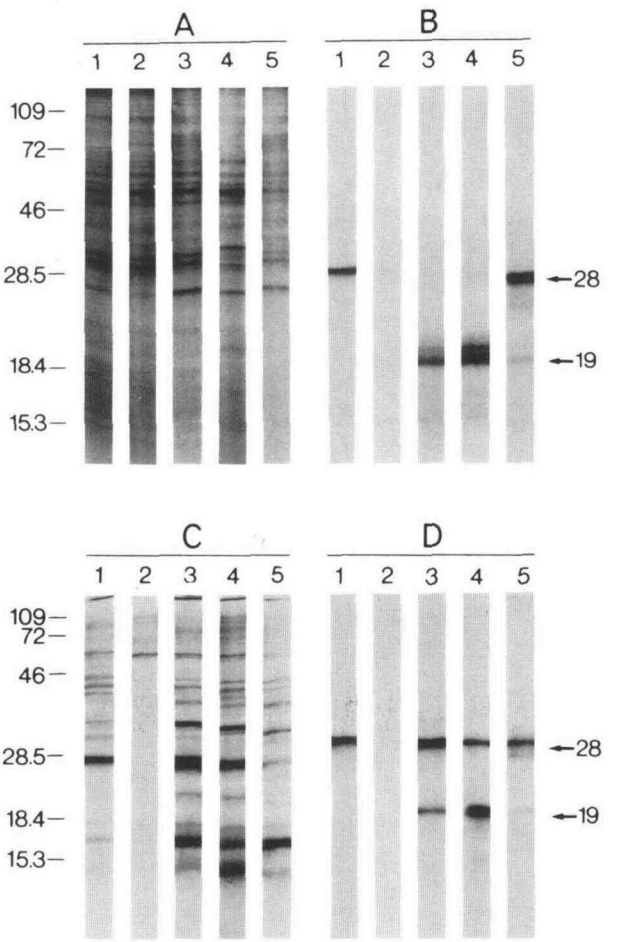

Figure 2. SDS-PAGE Autoradiographs of ^{35}S-Labeled Proteins in Cell Walls and Culture Media from Strains Variously Expressing Hydrophobin Genes and Concomitant Morphologies.

Lanes 1, *A41 B41*; lanes 2, *A41 B41 thn*; lanes 3, *A41 B41/A43 B43*; lanes 4, *Acon Bcon*; lanes 5, *Acon Bcon fbf*.
(A) Hot-SDS extract from walls.
(B) Subsequent formic acid extract oxidized with performic acid.
(C) Proteins precipitated from the medium with TCA.
(D) As given in **(C)** but oxidized with performic acid.
For comparison, the amounts loaded per lane were obtained from mycelia containing the same amounts of total TCA-precipitable radioactive material.

Peptide Sequencing

N-terminal amino acid sequencing of the oxidized 19-kD protein showed the sequence (Gly-Gly-Gly-Lys-Gly-Ala-Gly-Gln-Ala-X_{aa}-Asn-), as present in the deduced Sc4 protein (Schuren and Wessels, 1990) after splitting off the signal peptide between Pro^{20} and Gly^{21}. The two faster migrating proteins shown in Figure 3, lane 3, had the same sequence indicating differently modified forms of pSc4. The protein at the 28 kD position had the sequence ([Gly]-Gly-His-Pro-Gly-X_{aa}-X_{aa}-X_{aa}-Pro-) as present in the deduced Sc3 protein (Schuren and Wessels, 1990) after splitting off the signal peptide between Pro^{24} and Gly^{25} or between Gly^{25} and Gly^{26}. The glycine doublet at the N-terminal end was found in about 40% of the molecules. Further reading of the sequence was not possible, probably due to the presence of protein modifications. A second band visible just below the 28 kD band (Figure 3) showed the same sequence and thus represents a differently modified form of pSc3. In accordance with the deduced amino acid compositions, radioactive lysine was incorporated into the products of the *Sc4* gene but not into those of the *Sc3* gene, whereas the products of both genes were labeled by radioactive leucine (data not shown). We suggest that the 28-kD protein is a covalently linked dimer of pSc3.

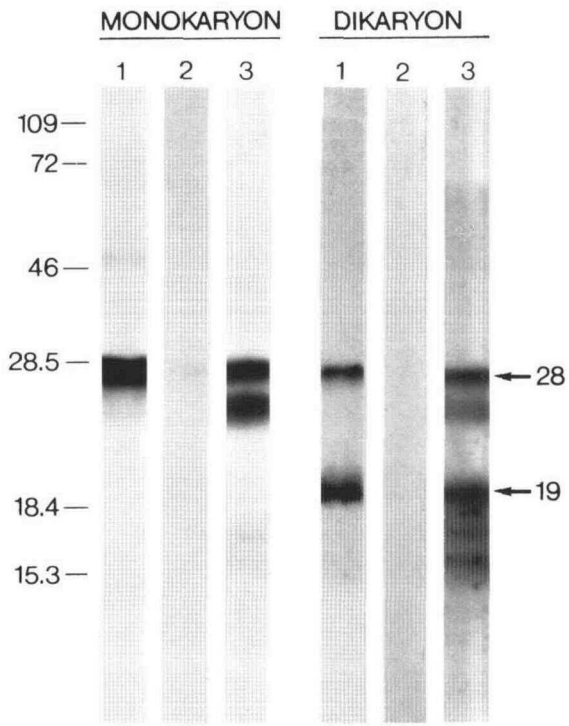

Figure 3. SDS-PAGE Autoradiographs of ^{35}S-Labeled Hydrophobins, after Oxidation with Performic Acid, from Monokaryon and Dikaryon.

Lanes 1, walls of emerged hyphae; lanes 2, walls of submerged hyphae; lanes 3, culture medium.
Emerged structures in the monokaryon were aerial hyphae, those of the dikaryon mainly fruit body primordia. The amounts of cell walls used for extraction and the amounts of medium in the comparisons were derived from fungal hyphae that had incorporated the same radioactivity into total proteins.

Emerging Hyphae Accumulate Hydrophobins in Their Walls

If the wall hydrophobins play a role in the emergence of aerial structures, one would expect their preferential occurrence in the walls of such structures. To obtain a sample of pure submerged hyphae, cultures were grown on a floating nylon net (see Methods), and hyphae protruding from the underside into the medium were collected. Such submerged hyphae from monokaryon and dikaryon contained very little of the hydrophobins in their walls and apparently excreted all hydrophobins into the culture medium (Figure 3). Because the mycelium growing in the nylon net probably also contained hyphae in direct contact with the culture fluid, this result does not exclude the possibility that submerged hyphae in this upper layer of the culture do contain hydrophobins in their walls.

DISCUSSION

We previously cloned nine Schizophyllum genes that are only activated in the dikaryon at the time of fruiting (Mulder and Wessels, 1986). Together these genes code for about 5% of the total mRNA mass, which is about the whole mass of unique mRNAs present in the dikaryon, but not in the monokaryon, at this stage of development (Hoge et al., 1982). In situ hybridizations have shown that these dikaryon-specific mRNAs accumulate only at the time and place of fruit body initiation (Ruiters and Wessels, 1989a), most abundantly in hyphae that are engaged in fruit body formation but to a lesser extent also in the supporting substrate hyphae (Ruiters and Wessels, 1989b). Although the concentration of these mRNAs then decreases in the vegetative mycelium and abortive fruit bodies, their concentration remains high in growing fruit bodies (Mulder and Wessels, 1986). Most abundant among these dikaryon-specific mRNAs are those derived from the *Sc1* and *Sc4* genes, which may accumulate to approximately 0.5 and 3.5%, respectively, of the total mRNA mass. The *Sc3* gene produces an abundant mRNA (up to 1% of the total mRNA mass) in both monokaryon and dikaryon but has a relatively low abundance in developing fruit bodies (Mulder and Wessels, 1986).

Sc1, *Sc4*, and *Sc3* are members of a gene family and encode similar small hydrophobic cysteine-rich proteins (Schuren and Wessels, 1990) that we call hydrophobins. However, they are under different genetic controls (see Table 1). *Sc1* and *Sc4*, but not *Sc3*, are controlled by the combinatorial activities of the mating-type genes *A* and *B* (Mulder and Wessels, 1986; Ruiters et al., 1988) and by the *FBF* gene (Springer and Wessels, 1989). All three genes are controlled by the *THN* gene (Wessels et al., 1991).

The abundance of the hydrophobin mRNAs is strongly correlated with development of the mycelia (see Figure 1 and Table 1). Low abundance of *Sc3* mRNA is correlated with the absence of aerial hyphae, low abundance of the *Sc1* and *Sc4* mRNAs with the absence of fruit bodies. To detect proteins encoded by these hydrophobin genes in vivo, our strategy has been to compare electrophoretic protein patterns produced by the various coisogenic strains differentially expressing these genes. Notwithstanding the large differences in hydrophobin mRNAs and expressed morphologies, only minor differences were seen when comparing SDS-extracted proteins on one-dimensional gels. However, differences in major proteins were seen in the culture media, as noted earlier for the monokaryon and dikaryon (de Vries and Wessels, 1984). Upon treatment of these medium proteins with formic acid and performic acid, dissociating hydrophobic interactions and possible disulfide linkages, a simple protein pattern arose indicating the products of the *Sc3* and *Sc4* genes as the major excreted proteins. Their identity was confirmed by

N-terminal amino acid sequencing. However, a major proportion of these hydrophobins is also retained by the cell walls, primarily by those of emerging hyphae, in which they form hot-SDS-insoluble complexes. Because performic acid dissociates these complexes, we originally thought that intermolecular disulfide bonds were responsible for their insolubility. However, dissociation also occurs by treatment with trifluoroacetic acid (O. M. H. de Vries and J. G. H. Wessels, unpublished data), which points to a major contribution of hydrophobic interactions between the hydrophobins in formation of the insoluble complexes. The dissociating effect of performic acid oxidation may have resulted from a decrease in the overall hydrophobicity of the molecules. However, besides hydrophobic interactions, the occurrence of other bonds contributing to insolubilization of the hydrophobins in the cell walls cannot be excluded at the moment.

At the time of emergence of aerial structures, synthesis of hydrophobins is substantial. From the labeling experiments, we calculate that at the fourth day of growth the monokaryon and the dikaryon were directing 5.6 and 8.1%, respectively, of their protein-synthesizing activities towards synthesis of the hydrophobins. We surmise that the insoluble hydrophobin complexes confer hydrophobicity to the walls of emerged hyphae and their incorporation into the walls may even be responsible for the tendency of the hyphae to leave the moist substrate and emerge into the air. In that case, the incorporation of the hydrophobins into the wall may be a major molecular switch during differentiation.

The hydrophobin genes are inactive in young colonizing hyphae (Mulder and Wessels, 1986; Ruiters and Wessels, 1989a). The first developmental switch would be the activation of these genes, *Sc3* in both monokaryon and dikaryon, *Sc1* and *Sc4* in the dikaryon only. The small hydrophobins are now excreted into the culture medium. A second developmental switch, possibly triggered by some environmental cue at the substrate/air interface, could then cause these hydrophobins to be retained in the wall determining these hyphae for emergence from the substrate. Hyphae containing pSc3 in their walls would differentiate aerial hyphae; those containing the less hydrophobic pSc1 and pSc4 in their walls would aggregate to form fruit bodies. A third regulatory mechanism appears to exist in the developing fruit bodies. The fruit body hyphae are high in *Sc1* mRNA and *Sc4* mRNA but low in *Sc3* mRNA (Mulder and Wessels, 1986) and contain little pSc3 in their cell walls (see Figure 3). Because all emerged hyphae are unable to take up nutrients, the first developmental switch would perform the important function of ensuring that a minimum amount of submerged feeding mycelium is formed before hyphae can acquire a tendency to leave the substrate.

Until now, experiments to test directly the assumed role of the hydrophobins in morphogenesis have been unsuccessful. Growing hydrophobin-producing strains and nonproducing regulatory mutants side by side on agar plates did not result in restoration of the normal phenotype in the mutants. This is perhaps not surprising because it is difficult to see how hydrophobins present in the medium could be incorporated into the walls of emergent structures of the mutants. Also, attempts to disrupt the hydrophobin genes by transformation with inactivated genes have met with no success. Apart from the possibility that homologous recombination is a rare event in this fungus, these experiments were hampered by the fact that the expected phenotypes arose with high frequency in control experiments because of the high mutation rates of the *FBF* gene (Springer and Wessels, 1989) and the *THN* gene (Schwalb and Miles, 1967).

The *rodA* gene of *Aspergillus nidulans*, implicated in the formation of the conidial rodlet layer, has recently been shown to have homology to the members of the Schizophyllum hydrophobin gene family (Stringer et al., 1991). In conjunction with the results reported in this paper, this suggests that the *Sc3* gene is responsible for the formation of the rodlet layer seen on Schizophyllum hyphal walls (Wessels et al., 1972). In addition, the homology between hydrophobin genes of an ascomycete and a basidiomycete indicates that a morphogenetic role for the hydrophobins as described for Schizophyllum may be of general significance in fungi.

METHODS

Strains, Culture Conditions, and Labeling

The strains of *Schizophyllum commune* shown in Table 1 have been described (Ruiters et al., 1988; Springer and Wessels, 1989) including the *thn* mutants that we isolated as frequently occurring variants of the monokaryons (Wessels et al., 1991). All mycelia were grown from a mycelial homogenate as a lawn on the surface of minimal medium (Dons et al., 1979) in continuous light at 24°C for 4 days. For RNA isolation, the mycelia were grown directly on medium solidified with 0.7% agar. For labeling experiments, the mycelia were grown for 3 days on water-permeable cellophane (330P, British Cellophane Ltd, Bridgewater, UK) overlying 0.7% agar medium. The mycelia growing on the cellophane membrane (diameter 4.8 cm) were then detached and floated onto 4 mL of liquid minimal medium containing 9.25 MBq mL^{-1} $^{35}SO_4^{2-}$ (specific activity 1000 to 1500 GBq mg^{-1}; $MgCl_2$ replacing $MgSO_4$ in the medium), and cultivation was continued for 24 hr. To isolate submerged hyphae, the mycelium was grown on a nylon net (50-μm mesh) floating on liquid medium (Mulder and Wessels, 1986). In this case the mycelium was labeled (277 kBq mL^{-1} $^{35}SO_4^{2-}$ in medium with 0.5 mg mL^{-1} $MgSO_4$) during the whole period of growth. Hyphae protruding from the underside of the net were scraped off (submerged hyphae) and the remainder

peeled off the net (emerged hyphae contaminated with submerged hyphae). All hyphal material was frozen in liquid nitrogen and stored at −70°C.

Quantitation of mRNAs

Total RNA was isolated, denatured, spotted onto GeneScreen-Plus membrane (Du Pont), and hybridized to ^{32}P-labeled cDNA clones (Mulder and Wessels, 1986; Ruiters et al., 1988). Radioactivity was measured by liquid scintillation counting. Hybridization to a clone of 18S rRNA served to compare the amounts of total RNA spotted.

SDS-Soluble Proteins

To the culture medium used in brief labeling experiments, 1 volume of the culture medium from a 4-day-old dikaryon was added for coprecipitation. The medium was then made 5% in TCA, and precipitated proteins were collected after 1 hr on ice. The precipitate was washed twice with 5% TCA containing 10 mM $MgSO_4$ and dissolved in SDS sample buffer (2% SDS, 0.05 M Tris-HCl, pH 6.8, 10% glycerol, 0.001% bromophenol blue) by heating at 100°C for 10 min. SDS-soluble mycelial proteins were obtained as follows: The mycelium was fragmented in an X-press (AB Biox, Göteborg, Sweden) at −25°C and the broken material quickly thawed in extraction buffer (0.1 M Tris-HCl, pH 8.0, 10 mM $MgSO_4$, 1 mM phenylmethylsulfonyl fluoride). To obtain total SDS-soluble proteins, the suspension was immediately brought to 5% TCA, the precipitate washed twice with 5% TCA, 10 mM $MgSO_4$, 1 mM cysteine, 1 mM methionine, then twice with cold acetone (−20°C), and dried. Proteins were then extracted with SDS sample buffer at 100°C for 10 min. For cell wall proteins, cell walls were prepared by centrifuging (1500*g*) the broken mycelium in extraction buffer and washing the pellet three times with extraction buffer and twice with water. The washed walls were freeze dried and then extracted with SDS sample buffer at 100°C for 10 min.

Dissolution of Proteins with Formic Acid and Oxidation with Performic Acid

Walls extracted with hot SDS, washed twice with water, and freeze dried, were subsequently extracted with 98% formic acid (100 μL [mg dry wt]$^{-1}$) in a sonicating water bath (Sonicor Instruments Corporation, Farmingdale, NY) for 1.5 hr, keeping the temperature below 10°C. After centrifugation, 2 volumes of performic acid were added to the supernatant, and oxidation was allowed to proceed on ice for 4 hr. Performic acid was prepared by mixing 1 volume of 30% H_2O_2 with 9 volumes of 98% formic acid and used after standing at room temperature for 1 hr. Formic acid extracts, before and after oxidation with performic acid, were diluted 20 times with water, freeze dried, and dissolved in SDS sample buffer. TCA-precipitated proteins from culture media were also dissolved in formic acid and oxidized with performic acid before being taken up in SDS sample buffer.

PAGE and Protein Sequencing

Electrophoresis was generally done in 12.5% polyacrylamide gels according to Laemmli (1970). For autoradiography, gels were soaked in 1 M sodium salicylate immediately after electrophoresis (Chamberlain, 1979), dried at 65°C, and exposed to preflashed film (Kodak X-Omat AR). For N-terminal protein sequencing, electrophoresis was done with the neutral buffer system MZE-3328IV of Jovin (1973), and gels were stored overnight before use to avoid N-terminal blockage during electrophoresis (Moos et al., 1988). Electroblotting was done on polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, Bedford, MA) by semi-dry transfer (Multiphor II Electrophoresis System, Pharmacia, Uppsala, Sweden) at 0.8 mA cm^{-2} for 2 hr. The blot was stained for 30 min in a fresh solution of 0.1% Coomassie Brilliant Blue R 250 in 10% acetic acid, 30% methanol, and destained in the same solvent for 3 hr. Protein bands of interest were excised, and amino acid sequencing was carried out with a pulse liquid sequenator on line connected to a phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA).

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