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# DEVELOPMENTAL REGULATION OF FUNGAL CELL WALL FORMATION

*J. G. H. Wessels*
Department of Plant Biology, University of Groningen, 9751 NN Haren, The Netherlands

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## INTRODUCTION

Cells of plants and fungi have cell walls,[1] but these walls mostly arise in different ways. Cells in multicellular plants typically arise in meristems and most walls are formed as cross walls between dividing cells, which thus remain firmly attached to each other. Subsequently, cells committed to differentiation enlarge their walls by diffuse extension growth. Fungal cells, in contrast, are not generated in meristems but grow as separate tubular cells (hyphae) that extend apically and regularly branch by forming new hyphal apices. A mycelium is thus formed in which hyphae may anastomose to produce a network of interconnected hyphae. Even when tissues are formed such as in fruit bodies, these arise by interactions of separate apically growing hyphae (174). In some fruit bodies, after differentiation of the various tissues, there is a rapid phase of expansion based on diffuse extension of cell walls, a process that is superficially similar to diffuse expansion in plant cells (80, 94). Conversely, some individually growing plant cells such as root hairs and pollen tubes display apical growth similar to fungal hyphae (145). In fact, just a century ago Reinhardt (115) firmly established the phenomenon of apical wall growth by studying the growth of both root hairs and fungal hyphae. In both cases apical

[1]The terms cell and cell wall will be retained throughout even though in many cases fungi are actually coenocytic



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growth is typically associated with invasive behavior of the cells but the basic mechanisms involved could be different. Many studies on apical growth of "fungal" hyphae are performed with oomycetes such as Achlya and Saprolegnia. Since these organisms (among which there are many plant pathogens) are only distantly related to true fungi (4, 31, 168), results obtained with these fungi in disguise should be used with caution.

The possession of walls allows fungi to generate turgor. This property, in combination with the excretion of the wall and of substrate-digesting enzymes at growing hyphal apices, enables hyphae to penetrate solid organic substrates by tunneling their way through digestible solids. This capacity provides them with a unique niche in nature and has probably allowed their separate evolution apart from plants and animals (175). For instance, saprotrophic fungi that produce lignin peroxidases and cellulases are the chief degraders of plant remains; without their activities plant life would eventually subside. Their ability to invade living plants and animals appears to be curtailed only by the defense mechanisms these organisms have developed. For humans, the importance of the immune system in this respect has recently been highlighted by the fate suffered by immunocompromised patients (105). Plants have developed various defense mechanisms and when these are breached by some (necrotrophic) fungi, death inevitably results. However, most plants have developed intricate symbioses with fungi, probably early in evolution. The importance of the mutualistic symbiosis called mycorrhiza can hardly be overestimated (59, 113, 114). However, the parasitic symbiosis, in which the plant suffers but is not killed, has received most attention because of its importance in agriculture.

The polarized activities of the fungal cytoplasm are critical to an understanding of fungal growth. The hyphal tip not only synthesizes a cell wall and excretes enzymes but also perceives chemical and physical signals that modify growth and development. However, the fungal mycelium is not a linear system but rather a network that allows for communication between different parts of the colony and translocation of water and nutrients. Thus, fungi have the ability to grow from a food base, be it a piece of dead wood or a living plant, through nonnutritive substrates (76, 112). Assimilation streams may also be redirected to allow for the emergence in nongrowing parts of new hyphae that excrete idiophasic enzymes or become involved in development of reproductive structures—most conspicuously, the fruit bodies of basidiomycetes (174).

In this review I survey what is known about the polarized excretion of the wall, including wall proteins, and how this can be related to phytopathogenic interactions. With respect to wall proteins I emphasize the emerging importance of the newly discovered hydrophobins. The review will not deal with the important role of cell-wall derivatives and excreted proteins in signaling to the plant the presence of the fungus (43, 123).

## BIOCHEMISTRY OF THE CELL WALL

Variation in the polymers that make up the walls of fungi allows the principles involved to be resolved by comparative biochemistry. For detailed accounts of the various polymers found in fungal walls the reader is referred to a number of comprehensive reviews (5, 8, 46, 120, 179).

### *Cell Wall Architecture*

The mature fungal wall is an elastic entity that resists hydrostatic turgor pressure, typically 0.5–2.5 MPa (see ref. 81). A microfibrillar component appears to resist stretching, and a matrix component between the microfibrils prevents compression of the wall. However, evidence indicates that most polymers in the mature wall are crosslinked by covalent and hydrogen bonds rather than existing as separate components.

The true fungi (chytridiomycetes, zygomycetes, ascomycetes, basidiomycetes) have chitin [(1–4)-β-linked *N*-acetylglucosamine], the oomycetes cellulose [(1–4)-β-linked glucose] as the major microfibrillar or structural wall component. Chitin may account for from 40% to 0.3% of the dry weight of the wall (96, 97, 138). Oomycetes also make chitin but the polymer may not play a structural role (24).

In the zygomycetes part of the chitin is deacetylated immediately after synthesis but before chains crystallize, and a polymer named chitosan is produced (2, 37). However, partially deacetylated glucosaminoglycans also arise (35). These insoluble polycationic polymers bind ionically to essentially soluble polyanionic glycuronans (containing glucuronic acid, fucose, mannose, and galactose) that are thereby maintained insoluble in the wall (35, 36).

In the walls of ascomycetes and basidiomycetes most of the chitin (or more precisely the glucosaminoglycan) is fully acetylated and associated with (1–3)-β-/(1–6)-β-glucan in an alkali-insoluble complex. Like the glycuronan in the walls of zygomycetes, the glucan is essentially soluble in water and/or alkali but apparently covalently linked to the alkali-insoluble glucosaminoglycan (79, 95, 96, 136–138, 149). Little is known about the linkage between the two polymers; in one case amino acids—particularly lysine—were involved (136), whereas direct linkage between the glucan and chitin was indicated in another (149). These highly insoluble complexes are difficult to dissect chemically. Irrespective of the kind of covalent linkage involved, it has been shown that in the basidiomycete *Schizophyllum commune* (142, 180) and in the ascomycete yeast *Saccharomyces cerevisiae* (62) the glucan in the complex is synthesized as a water/alkali-soluble precursor glucan that is secondarily linked to the glucan. Therefore, no alkali-insoluble glucan can be formed when chitin synthesis is inhibited (40, 45, 142). The alkali-soluble precursor glucan appears to contain (1–3)-β-linkages only. How and when the (1–6)-linked

glucan branches are attached is unknown, but (1–6)-β-linked single-glucose branches may be attached by a recently discovered glucosyltransferase (61). Unsubstituted (1–3)-β-linked glucans interact by hydrogen bonding to form triple helices (75, 88) but those bearing single-glucose branches have this same property (125). Consequently, the alkali-insoluble complex may be a strongly crosslinked composite, with hydrogen bonds between homologous chains and covalent bonds between heterologous chains.

Because the linkage between chitin and glucan resists alkali treatment, the chitin-glucan complex was recognized as an entity in the wall. Components extracted by alkali may have been linked to other wall components by alkali-labile bonds, including hydrogen bonds. For example, the high-mannan proteins that cannot be extracted from the wall of yeasts by hot SDS are released when the chitin-glucan complex is degraded by chitinase and/or (1–3)-β-glucanase (26, 44). There is evidence that these mannoproteins play a structural role and can self-assemble into high-molecular weight aggregates (30).

Proteins generally do not have a structural role in the wall. Some excreted proteins may be simply caught in the wall fabric during excretion, but others may have important roles in modifying and crosslinking the wall polymers. Proteins exposed to the outer surface may play an important role in determining antigenic and adhesive properties (28, 63). The possible role of surface polymers of phytopathogenic fungi in adhesion to plant surfaces and in recognition phenomena has been extensively discussed (34, 91). With respect to proteins, the fimbriae seen at the surface of phytophathogenic fungi (38, 151) are of interest but their role is not yet clear. Later in this Review I would like to propose a role of members of the hydrophobin family in these processes.

## *Cell Wall Synthetic Enzymes*

Of the wall polymers mentioned above, the proteins (including the mannoproteins) are clearly synthesized in the cytoplasm on the endoplasmic reticulum and directed to the cell surface by secretory vesicles via the Golgi apparatus (oomycetes) or Golgi equivalents (true fungi) (87, 126, 156). There is evidence that (1–6)-β-glucan chains are made within the cytoplasm. Bussey and coworkers (13, 20) have identified in *Saccharomyces cerevisiae* several killer-toxin-resistant mutants defective in (1–6)-β-glucan synthesis. Their findings suggest that this polymer is synthesized sequentially and involves the products of several (*KRE*) genes, some of which are in the secretory pathway, while others are cytoplasmic or membrane proteins.

Autoradiography has shown that chitin (162) and (1–3)-β-glucan (48) are directly deposited outside the plasma membrane. Similar studies but using isolated plasma membranes and nucleotide sugars have shown that these polymers are indeed vectorially synthesized by integral plasmalemma proteins (25, 71, 134).

The properties of chitin synthase have received most attention, undoubtedly because enzyme preparations displaying this activity are easily prepared and produce large amounts of crystalline chitin when presented with the substrate uridine-diphospho-*N*-acetylglucosamine (UDPGlc*N*Ac) together with an activator such as *N*-acetylglucosamine and $Mg^{2+}$ (53). Such enzyme preparations are usually made from mixed membrane or purified plasmalemma (25, 164) or chitosomes. The latter are cytoplasmic particles containing chitin synthase plus a variety of other proteins and lipids (6, 17, 49, 78). A brief proteolytic digestion stimulates chitin synthase in these preparations, particularly that of chitosomes which are otherwise inactive (6, 27). Whether proteolytic activation plays a role in vivo is unknown; it has been suggested that a chitin synthase responsible for synthesis of most of the chitin in *Saccharomyces cerevisiae* (chitin synthase 3) is nonzymogenic (109, 133). When partially delipified, chitin synthase can be stimulated by adding phospholipids (42, 100, 165), which suggests that lipids have a role in regulating enzyme activity. Chitosomes may insert inactive chitin synthase in the plasmalemma where the lipid environment activates the enzyme.

It takes several minutes for the synthesized chitin chains to crystallize in vitro (167)—a transient state of chitin that also occurs in vivo (166). During this interval the modifications such as deacetylation and linkage to β-glucan mentioned above probably occur and then impede further crystallization. Indeed, chitin in the wall is poorly crystalline in contrast to chitin synthesized in vitro or synthesized in vivo on regenerating protoplasts before crosslinking to β-glucan (161).

Three different chitin synthase genes have been cloned from *Saccharomyces cerevisiae* (21, 22, 139). Only disruption of all three genes leads to a lethal phenotype (133), which suggests that these genes can substitute for each other. More than one chitin synthase gene has also been identified in other fungi (15). It is hoped that the cloning of these genes or purification of the chitin synthase proteins will facilitate the preparation of specific antibodies by which the cellular location of the chitin synthases can be established.

Fungal membrane preparations also readily synthesize (1–3)-β-glucan with uridine-diphospho-glucose (UDPGlc) as a substrate and GTP as an activator (71, 134, 153, 169). The (1–3)-β-glucan synthesized in vitro (169) and in vivo on regenerating protoplasts (83) is microfibrillar and crystalline. However, as with the product of chitin synthase, a transient state of noncrystallinity may occur that allows for modifications of the glucan, e.g. formation of (1–6)-β-linked branches and linkage to glucosaminoglycans (chitin). Monoclonal antibodies have been prepared that inhibit both (1–3)-β- and (1–4)-β-glucan synthases of Saprolegnia in vitro (47). These antibodies bind preferentially to the plasmalemma of protoplasts released from apical cells, which suggests localization of these enzymes in growing apices only.

# CELL WALL BIOGENESIS IN RELATION TO MORPHOGENESIS

## *The Polarized Activity of the Cytoplasm*

Reinhardt's finding (115) that the wall expands at the hyphal apex was confirmed by microautoradiography of chitin and glucan synthesis (7, 52, 180). Although it is now generally accepted that the nascent wall over the apex must be plastic in order to expand, and rigid or elastic subapically to resist turgor pressure, Reinhardt thought that the wall had uniform strength over the apex. He reached this conclusion because an experimental increase in turgor did not cause the wall to rupture at the extreme apex, but resulted in bulging and rupture of the wall at the base of the extension zone where circumferential stress in the wall becomes maximal. It is now becoming apparent that the apical cytoplasm is rich in cytoskeletal elements and may have a firm structure that protects a delicate wall over the apex against high turgor pressure (72, 111, 171). The pressure-induced bursting of the wall at the base of the extension zone may be explained because here the wall is less protected by the structured cytoplasm (172). Disruption of the apical F-actin in growing tips of *Saprolegnia ferax* by UV irradiation induces bursting of the wall at the very apex (74).

Of the cytoskeletal elements most attention has gone to actin, which is highly concentrated at growing hyphal apices (65, 67, 121, 124), at the expanding poles of fission yeasts (89), and in the buds of budding yeasts (1). In contrast, microtubules do not seem to play a prominent role in the polarized activities of fungal cells (70, 108). Actin is thought to play a multifunctional role by coordinating cell-wall synthesis, cytoplasmic migration, and organelle positioning (66). As in animal cells (19), the polarized actin in fungal apices is likely connected to plasmalemma proteins and via these to the extracellular matrix, i.e. the cell wall. Thus, based on evidence obtained with animal cells, apical actin is hypothesized to be involved in vesicle fusion (111), anchoring wall synthetic enzymes in the plasmalemma (170), and positioning or excluding ion channels. Some fungal systems are providing additional information. For instance, during budding in *Saccharomyces cerevisiae,* the degree of actin polarization varies during the cell cycle, depending on the activities of different cyclins in association with the product of the *CDC28* gene, a protein kinase (86). Importantly, the distribution of actin correlates with the sites of wall expansion and exocytosis. A mutation in the *cot-1* gene of *Neurospora crassa* causes restricted hyphal extension (colonial growth). The gene was cloned and encodes a putative cAMP-dependent protein kinase (185). How protein phosphorylation is involved in actin localization remains unclear.

Fungal hyphae, like other polarized systems, drive electrical current through themselves, mostly positive charge flowing in the apex and out of the trunk (55, 60). Under certain nutritional conditions or dependent on the age of the mycelium, however, the current may stop or be reversed without effect on extension growth of the hypha. This interruption or reversal considerably weakened the idea that electrical currents play a role in establishing polarity, e.g. by processes like electrophoresis of vesicles or membrane proteins (see ref. 170). On the other hand, a flow of protons into the apex and out of the subapical part of the hyphae may always accompany apical growth, since it constitutes only one component of the electrical currents observed. The proton gradient was postulated by Slayman & Slayman (141) who observed that the plasmalemma at the apex is depolarized relative to that in subapical parts. This depolarization is thought to be caused by the exclusion of proton pumping ATPase activity from the apical plasmalemma so that protons are subapically pumped out of the hypha and are replenished by protons that flow into the apex, probably in conjunction with other ions and nutrients (140). Indeed, measurements with a pH-sensitive microelectrode have shown the expected pH gradient around the growing hyphae (55, 60) and within the cytoplasm (117, 159). Strong support for such a proton flow has been presented by Belozerskaya & Potapova (11). These workers showed that isolation of the apical compartment from the subapical proton-pumping region causes a marked depolarization of the apical compartment, suggesting an intracellular flow of protons from the apex down through septal pores. Because the plasmalemma ATPase is estimated to consume 25–50% of the cellular ATP (140), these authors propose that the proton current serves the transfer of energy from distal parts of the hypha to the rapidly growing apex.

The possible involvement of $Ca^{2+}$ gradients in tip growth of fungi has been widely discussed (reviewed in ref. 73). The best evidence for such a gradient comes from studies on the oomycete *Saprolegnia ferax* (73), but in the true fungi the issue is not at all clear. In fact, the bases on which many observations have been made with fungi are subject to severe criticism (186).

Of great interest is the presence of mechanosensitive ion channels in the plasmalemma of fungi, first observed in the plasmalemma of *Saccharomyces cerevisiae* (57). These channels, passing both cations including $Ca^{2+}$ and anions, are activated by stretching the membrane in patch-clamp experiments, and have been implicated in regulating turgor. Such channels were also found in *Uromyces appendiculatus* and implicated in the formation of appressoria (157a, 188). When a germ tube encounters a stomatal ridge (or a ridge on an artificial surface) a small indentation is formed in the growing apex, possibly causing stretching of the membrane, opening of ion channels and signaling formation of the appressorium. Stretch-activated $Ca^{2+}$ channels were also detected in *Saprolegnia ferax,* apparently located in the apical plasmalemma

(50). In this case they were implicated in maintaining the $Ca^{2+}$ gradient in the apical cytoplasm necessary for hyphal extension. As pointed out by Morris & Sigurdson (101), the discovery of mechanosensitive ion channels raises the interesting prospect that the activities of other integral membrane proteins, not necessarily measurable by patch clamp, could be influenced by membrane tension. Hypothetically, for instance, mechanosensitive proteins in the plasmalemma continuously report the yielding of the wall over the apex during advance of the apical cytoplasm, and thus precisely regulate the activities of wall-synthesizing enzymes to maintain uniform wall thickness (172).

## *The Role of Turgor*

Although Reinhardt (115) concluded that turgor does not drive hyphal extension, later observations similar to his on living hyphae (110, 116) have been taken as evidence that turgor pressure is the driving force of extension. Consequently, the hypha has been treated as a wall-bound structure filled with a fluid under hydrostatic pressure. This generalization has facilitated the construction of mathematical models to explain the generation of a tubular wall by extension at one end (see ref. 170). However, when careful measurements were made, the predicted simple relationship between turgor and extension rate was not always found (see ref. 81). One complication is that most fungi rapidly equilibrate with imposed osmotic stress by importing or synthesizing osmotically active molecules and thus maintain a certain turgor pressure independent of the osmotic potential of the medium in which they grow (77). Recently, work on Saprolegna and Achlya has even resurrected Reinhardt's idea that turgor plays no role at all. Money & Harold (98, 99) found that these oomycetes do not regulate turgor and continue to grow at turgor pressures close to zero—at least *Saprolegnia ferax* maintains its normal hyphal shape. They also observed that the pressure needed to burst the apex (by injecting oil from the pressure probe used to measure turgor) declined proportional to turgor pressure, suggesting that growth rate was maintained by making a softer wall at the apex. In contrast, Kaminskyj et al (81) observed turgor regulation in *Saprolegnia ferax,* but likewise concluded that apical extension is largely independent of turgor. These oomycetes, which thrive in fresh water (water molds), could well be unusual in their osmotic behavior. Nevertheless, these studies indicate the importance of the apical cytoplasm in apical growth and morphogenesis, and possibly also apply to the true fungi.

Turgor probably plays an important role in the expansion growth of agaric fruit bodies (mushrooms) in which cell walls expand over their whole surface (80, 94). As in herbaceous plants, turgor also maintains the stiffness of these multicellular structures. In fruit bodies such as those of *Schizophyllum commune* and many polypores, the structure of the wall and the deposition of lignin-like polymers (23) provide support as in woody plants.

Another important role of turgor relates to the capacity of fungi to penetrate solid substrates. Excreted enzymes that digest the substrate may indeeed play a role (91), but obviously the hypha must be turgescent in order to invade even a partially digested substrate. Hyphae can penetrate inert substrates purely by mechanical force (92). After attachment of the appressorium of *Magnaporthe grisea,* the infection peg can puncture hard synthetic membranes, obviously without the need of enzymes, while turgor pressure in the appressorium was estimated to exceed 8 Mpa (80 bars) (69). During appressorium maturation melanin is deposited in the appressorial wall; this deposition may strengthen the wall and/or decrease its porosity for water. The functional significance of melanin deposition is indicated by the fact that melanin-deficient mutants are unable to penetrate leaf surfaces (33). In Uromyces, stomatal cuticular lips or ridges on artificial membranes that induce appressorium formation are flattened by the attached developing appressorium (90, 157), probably because of the turgor (estimated as 0.35 Mpa) of the appressorium.

## *Assembly of the Wall at the Apex*

The assumed plastic nature of the wall over the apex of growing hyphae would suggest structural differences between this wall area and the hardened subapical wall that must resist turgor pressure. Such differences have been observed by electron microscopy of apices of germlings of *Schizophyllum commune* that were either growing or not growing at the time of fixation (166). In contrast to the subapical wall, the wall over the growing apex contains nonfibrillar chitin with a high susceptibility to chitinase and hot dilute mineral acid, suggesting noncrystallinity of chitin in this area. This characterization agrees with the known noncrystallinity of chitin immediately after synthesis in vitro (167). The wall of apices that arc not growing at the time of fixation has the same structure as that of the subapical wall (166). That other workers showed microfibrils in the wall over apices of other fungi may be attributable to their use of wall preparations instead of chemically extracted intact hyphae. The wall over the growing apex is extremely fragile and is removed when hyphae are broken to make wall preparations; only in nongrowing hyphae does the apical wall remain intact (180). It was established by light microscopic autoradiography of germlings of *S. commune*, pulse-labeled with radioactive wall precursors followed by a chase, that the chitin synthesized at the apex is alkali-insoluble, but that all the glucan synthesized at the apex is soluble in alkali (180) and contains 1–3 linkages only (135). As the radioactive wall falls behind the apex during the chase, the glucan becomes alkali-insoluble by linkage to chitin and (1–6) linkages appear in the glucan. The moment that growth stops, these changes also occur in the apical wall.

The results described above were interpreted as follows (170, 178, 180): (1–3)-β-glucan (alkali-soluble) and chitin (alkali-insoluble) are extruded in a

steep gradient at the apex as free chains interacting with water and constituting a plastic mixture of polymers that can be easily deformed by the advancing cytoplasm. With time glucan chains become linked to chitin chains whereas homologous chains interact by forming hydrogen bonds. At the same time (1–6)-β linkages are introduced in the glucan. The crosslinking of wall components gradually hardens the wall—as occurs during the manufacturing of two-component composites. This process has been dubbed the "steady-state theory of apical wall growth" (170) because it asserts that during hyphal growth a steady-state amount of plastic wall material is present at the apex. Cessation of extension leads to hardening of the wall over the apex, which then becomes structurally, and probably mechanically, identical to the wall in subapical regions. The continuous advance of the hypha thus depends on the steep gradient in wall synthesis. If the gradient in synthesis were less steep, as in bud growth in yeasts, expansion would proceed for some time but hardening would eventually spread through the whole wall and growth would cease, necessitating recurrent initiation of buds (172). In fact, the general theory of a plastic wall undergoing hardening with time can explain the generation of any form of fungal cells, including formation of infection structures such as appressoria and haustoria, on the basis of differences in the spatial activities of exocytosis and plasmalemma-bound synthases. Note that this theory of wall growth can be applied to fungi with widely different wall polymers. The only requirement would be that these polymers are gradually crosslinked after synthesis. For instance, in the zygomycetes this could be accomplished by deacetylation of chitin and ionic interactions with glycuronans.



## *Passage of Proteins Through the Wall at the Apex*

Exocytosis of proteins and wall expansion are apparently coupled. In yeast this is suggested by the fact that several temperature-sensitive *sec* mutants are blocked in both protein secretion and surface growth at the nonpermissive temperature (126). In filamentous fungi the ultrastructural evidence showing exocytotic vesicles concentrated at the growing hyphal apex (51, 54, 56) is highly suggestive, although the many fusion profiles seen after conventional fixation are not seen after ultrarapid fixation and freeze substitution (68). We have shown that excreted proteins do indeed leave the mycelium at the apices of growing hyphae only, both during primary growth (183) and during the idiophase when growth of the mycelium as a whole has ceased (102, 103).

The process whereby proteins are extruded together with wall polymers at the growing apex suggests a mechanism by which these proteins could traverse the wall. A wall volume added at the extreme apex will be pushed to the outside of the wall while falling behind the tip, being stretched and crosslinked. Proteins excreted apically would thus traverse the wall by bulk flow without going through pores (172). Proteins could easily diffuse from the most

stretched outer-wall region into the medium, unless they are crosslinked to the wall (128), form insoluble complexes like hydrophobins do (see below), or encounter a relatively impermeable outer-wall component (107). This bulk flow theory (172) would solve the paradox of the porosity of the fungal wall not permitting the passage of large proteins (32, 127, 158).

The excretion of enzymes at the growing apex is clearly of great functional significance for saprotrophic fungi. The enzymes not only clear the way for penetration but they also ensure that the breakdown products are immediately available as nutrients for the most active part of the mycelium. In laboratory cultures, some enzymes, such as those involved in lignin degradation are only produced by secondary apices after primary growth has ceased (102, 103). However, in nature invasive growth probably occurs under nutrient-limiting conditions and the invading hyphae themselves may excrete the lignolytic enzymes. With respect to phytopathogenic fungi, controversy exists as to the role of excreted enzymes in penetration of host cells (reviewed in ref. 91). For instance, a large body of evidence supports an obligatory role of cutinase in breaching the cuticula of epidermal cells by *Nectria haematococca* (82). However, disruption of the single cutinase gene in this fungus had no effect on virulence or pathogenicity (144). Likewise, disruption of a cutinase gene in *Magnaporthe grisea* (152) and an endo-galacturonase gene in *Cochliobolus carbonum* (132) was without effect on pathogenicity. Maybe mechanical force, as demonstrated in *M. grisea* (69), can overcome the absence of enzyme activity. There may also be genetic back-up systems, as shown for the chitin synthases of yeasts discussed earlier. Alternatively, the enzymes under investigation may be members of a whole battery of enzymes conducive to penetration and loss of a single enzyme is of minor importance (91).



## HYDROPHOBINS IN MORPHOGENESIS AND PATHOGENESIS

### *Nature and Properties of Hydrophobins*

Hydrophobins were first discovered as the products of genes abundantly expressed during the emergence of fruit bodies and aerial hyphae in the basidiomycete *Schizophyllum commune* (41, 104). Sequencing of some of these genes and the corresponding cDNAs revealed that at least three belonged to a family putatively encoding small moderately hydrophobic proteins (about 100 amino acids) with signal sequences for secretion and eight cysteine residues characteristically spaced (131). These proteins identified in the hyphal walls of aerial structures (176, 177) were dubbed "hydrophobins," a name earlier used to denote any hydrophobic substance covering microbial cells (118). Since the publication of these hydrophobin sequences (131), a number of genes expressed abundantly by other fungi have been identified as hydrophobins by sequence comparison

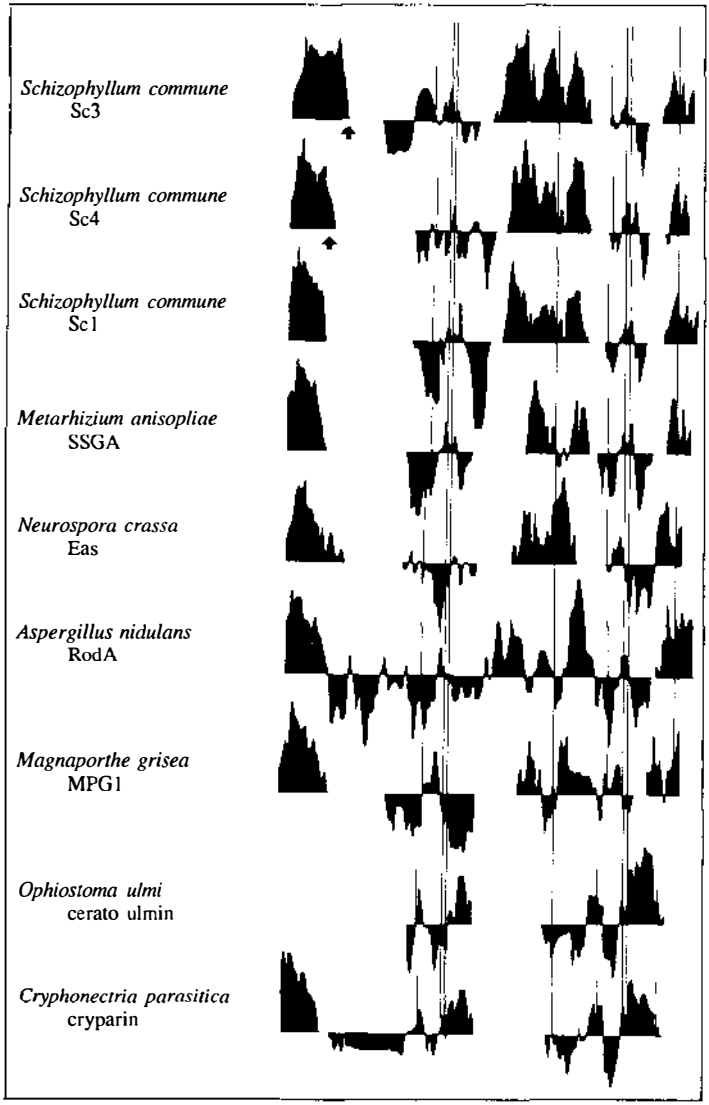


*Figure 1* Comparison of hydropathy patterns of Sc3, Sc4 and Sc1 from *Schizophyllum commune* (131), SSGA from *Metarhiziumanisopliae* (146), Eas from *Neurospora crassa* (10, 85), Rod A from *Aspergillusnidulans* (147), MPG1 from *Magnaporthe grisea* (155), cerato-ulmin from *Ceratocystis ulmi* (12), and cryparin from *Cryphonectria parasitica* (187). The patterns were determined using the parameters of Kyte & Doolittle (84). A six-amino acid window was used and plotted against position in the deduced amino acid sequence. The hydropathy patterns were then aligned around the first and second cysteine doublet and around the fourth and eighth cysteine residue, leaving gaps in the sequences where the hydrophobic regions (above the lines) alternate with hydrophillic regions. The hydrophobic amino-terminal sequence serves as signal sequence for secretion (the amino-termini for the mature hydrophobins, when known, are indicated by arrows); for cerato-ulmin only the sequence of the mature protein is given. Note that the first seven hydrophobins (class I) have similar hydropathy patterns that deviate from those of the two last hydrophobins (class II).

(Figure 1). However, at the nucleotide level the sequences of the hydrophobin genes are quite divergent; even the three hydrophobin genes of *S. commune, Sc3, Sc4* and *Sc1*, have only 45% homology in the coding sequences and do not cross-hybridize. At the amino acid level, the homologies are better if conservative substitutions are allowed, yielding 39% identity and 41% similarity. However, if the RodA protein of *Aspergillus nidulans* and the Eas protein of *Neurospora crassa* are also considered, the identity between the five hydrophobins drops to 11% and the similarity to 23% (these comparisons include signal sequences). Nevertheless, if the eight cysteine residues in all the proteins are aligned, a striking similarity of the hydropathy patterns can be seen for the hydrophobins listed in Figure 1, except for cerato-ulmin and cryparin. Because cerato-ulmin was recently classified as a hydrophobin (148), the two latter proteins may be called class II hydrophobins to distinguish their hydropathy pattern from the class I hydrophobins.

Apart from the class II hydrophobins, which were first identified as abundantly excreted proteins, the class I proteins listed in Figure 1 were all identified as the putative products of genes abundantly expressed at certain stages of development of the fungus. Of these, only the proteins encoded by the *Sc3* and *Sc4* genes of *S. commune* have been identified to date (176, 177). They can be detected as monomers in the medium of standing cultures, but only if this medium is subjected directly to electrophoresis. Precipitation of the proteins occurs upon shaking the medium and converts the monomers into SDS-insoluble aggregates. The proteins can also be found as SDS-insoluble aggregates in hyphal walls from aerial structures, but not in those of submerged hyphae. The SDS-insoluble aggregates can be solubilized in cold 100% formic acid and can then be visualized with SDS-PAGE as monomers, particularly after an oxidative treatment with performic acid, which prevents reaggregation (176, 177). Alternatively, they can be solubilized and dissociated with cold 100% trifluoroacetic acid (TFA) (39). The Sc3p and Sc4p hydrophobins are therefore not seen in hot-SDS extracts of the mycelium, even though they may constitute up to 10% of all the protein made by the fungus (39, 176). If this applies to all class I hydrophobins, then it is not surprising that these proteins, notwithstanding their abundance, have gone undetected till gene cloning revealed their existence. In fact, the insolubility of the hydrophobin assemblages in SDS can now be used to advantage. After extraction of most proteins by a hot-SDS solution, only hydrophobin-like proteins are left in the hyphal walls of a variety of fungi belonging to different classes (39).

The Sc3p hydrophobin of *S. commune* was purified and some of its properties determined (182). It spontaneously assembles into an SDS-insoluble membrane when confronted with a water-gas interface. When a solution of Sc3p is shaken or gases are bubbled through the solution, Sc3p immediately coats gas bubbles with a 10-nm thick amphipathic insoluble membrane with a typical rodlet mosaic displayed at the hydrophobic (gas) side. When a solution of Sc3p is

dried down on a hydrophillic surface the mosaic of rodlets facing the aerial side of the membrane can be directly observed after shadowing. This side of the Sc3p membrane displays a hydrophobicity similar to that of the surface of the artificial polymer teflon (contact angles of 1 µl water droplets about 110°). Since the Sc3p-containing walls of aerial hyphae display a surface with similar rodlets and hydrophobicity, this indicates that an insoluble Sc3p membrane coats these hyphae (182). This membrane was recently visualized with gold-labeled Sc3p-specific antibodies (181).

The Sc3 hydrophobin not only self-assembles at a water-air interface but also at interfaces between water and oils and water and a variety of hydrophobic solids (HAB Wösten & JGH Wessels, unpublished data). This capacity for self-assembly suggests that hydrophobins could also serve as an adhesive between hydrophillic and hydrophobic surfaces, such as the fungal wall and the hydrophobic surfaces of insect or plant hosts. For the Sc3p hydrophobin (the properties of the other class I hydrophobins listed in Figure 1 have not yet been determined), we surmise that the confrontation of the monomer with a hydrophillic/hydrophobic interface causes a conformational change in the protein and results in formation of a stable two-dimensional assemblage (a membrane) in which polar groups face the hydrophillic, apolar groups the hydrophobic phase. The conformational change can be likened to that occurring during interfacial activation of lipases (163).

## *Hydrophobins in Development*

Since work on the regulation of hydrophobin genes and their role in emergent growth of *Schizophyllum commune* has been recently reviewed (173–175) only a brief account of this work suffices. *S. commune* is the only fungus from which different hydrophobin genes (*Sc3, Sc4, Sc1*) have been cloned. The *Sc3* gene is located on chromosome X, the *Sc1* and *Sc4* genes on chromosome I (3). The three hydrophobin genes are silent in young cultures but become active at the time of emergent (aerial) growth (104, 130), except in mycelia that carry the *thn* mutation associated with formation of few aerial structures (177). In a monokaryon of *S. commune* only the *Sc3* gene is switched on. The Sc3 hydrophobin is then excreted into the medium by the growing submerged hyphae (177); this excretion occurs at growing hyphal tips (181). In the aerial hyphae that now emerge the hydrophobin occurs as an insoluble complex. We presume that in hyphae that breach the medium-air interface, the Sc3 hydrophobin monomers, driven to the surface by apical wall growth, self-assemble at the interface with air as in vitro (see previous section). From the top down the aerial hyphae are thus coated with an insoluble amphipathic Sc3p membrane exposing its rodlet-decorated hydrophobic surface to the outside (181).

The hydrophobin genes *rodA* and *eas* of *Aspergillus nidulans* and *Neuro-*

*spora crassa*, respectively, code for proteins (Figure 1) that may similarly form the hydrophobic rodlet layers on the conidiospores of these fungi. The *rodA* gene codes for a transcript under control of the *brl A* gene, a primary regulator of conidiogenesis in *A. nidulans* (147). The *rodA* transcript was not found in submerged hyphae nor in mature conidia but accumulated in the phialides that form the conidia. The only effect seen after disruption of the *rodA* gene was the disappearance of the hydrophobic rodlet layer on the conidiospores (147). The *eas* gene of *N. crassa* was independently identified as a gene (*bli-7*) that encodes a transcript abundantly expressed during blue light-induced conidiation (85) and as a gene (*ccg2*) encoding an abundant circadian-clock-controlled transcript (10). In both cases it was shown that disruption of the gene caused a phenotype known from a mutation in the *eas* (*eas*ily wettable) gene characterized by the absence of the hydrophobic rodlet layer on conidiospores (9), the targeted mutations being allelic to *eas*. Although these genetic studies do not prove that the proteins encoded by *rodA* and *eas* are solely responsible for formation of the hydrophobic rodlet layer, the in vitro experiments with the Sc3p hydrophobin of *S. commune* suggest this to be the case.

The *Sc1* and *Sc4* hydrophobin genes, in addition to the *Sc3* gene, are switched on in the dikaryon of *S. commune*. Activation of *Sc1* and *Sc4* requires the interaction of different *MATA* and *MATB* genes (104) or constitutive mutations in both these mating-type genes (119). In addition, at least one wild-type copy of *FBF* (143) and *THN* (177) must be present. A number of other genes, among which the *Sc7* and *Sc14* genes, which code for wall proteins with a surprising homology to pathogenesis-related proteins of plants (PR1) (129), are similarly regulated and their expression also correlates with the emergence of fruit bodies. Sc4p and Sc7p were identified and found to be excreted by submerged hyphae at the time of fruit-body formation. Like Sc3p in aerial hyphae, the Sc4p hydrophobin is present as an SDS-insoluble TFA-extractable complex in the walls of hyphae that constitute the fruit-body tissue (176), whereas the hydrophillic Sc7p is only loosely bound to the walls (129). We recently observed by immunocytochemistry that the Sc3p hydrophobin is excluded from the Sc4p- and Sc7p-containing hyphae of the inner tissue of the fruit bodies, but is present in the walls of the air-exposed hyphae that cover the fruit bodies and in the hymeneal hyphae (SA Ásgeirsdóttir & JGH Wessels, unpublished data).

Although the Sc4p and Sc3p hydrophobins share some properties in vitro, we do not yet know what induces the Sc4p hydrophobin to assemble in the walls of the fruit-body tissue nor what its function is. One possibility is that Sc4p is instrumental in adhesion of the hyphae to each other. Something at the surface of hyphae that engage in formation of hyphal aggregates in fungi, such as fruit bodies, cords and rhizomorphs, must induce these hyphae to stick

to each other and hydrophobins would appear to be good candidates to fulfil this task.

## *Hydrophobins in Adhesion and Pathogenesis*

The extensive literature on adhesion of fungi to the hydrophobic surfaces of plants and arthropod cuticles has been recently reviewed (14, 91, 106). Hydrophobic interactions are thought to occur between the hydrophobic surface of airborne spores and the host surface. Hydrophobins are probably involved, at least in cases where rodlets are observed on the spores. Active adhesion of spores is also observed. For instance, upon hydration spores of *Magnaporthe grisea* expel a preformed material from the site of future germ tube formation by which they tightly adhere to hydrophobic surfaces (58). In addition, the germ tube itself and the appressorium from which the infection peg must penetrate the epidermis must tightly adhere to the hydrophobic surface.

The fact that the interface between a hydrophillic and a hydrophobic material is sufficient to trigger the assembly of the Sc3p hydrophobin into an amphipathic membrane (182; HAB Wösten & JGH Wessels, unpublished observations) suggests that hydrophobins with similar properties would be ideally suited for establishing adhesion between the hydrophillic fungal wall of the fungus and the hydrophobic host surface. If a hypha grows over a surface, excreting at its growing apex a hydrophobin, like Sc3p, one would expect self-assembly of the hydrophobin not only at the side of the hypha facing the air, but also at the side facing the solid surface if it is hydrophobic. The hydrophillic wall would thus become firmly glued to the hydrophobic surface by an insoluble amphipathic membrane, even when wet. The beauty of this system is its simplicity. The hydrophobicity of the surface would be sensed by a molecule at the outer wall surface that itself subsequently serves as an adhesive to glue the two incompatible surfaces together. Adhesion could then generate signals, e.g. via mechanosensitive ion channels in the plasmalemma, that trigger morphogenesis of infection structures (157a). Although still speculative, recent investigations lend some support to such a role of hydrophobins.

During nutrient deprivation the insect pathogen *Metarhizium anisopliae* produces haustoria and cuticle-degrading enzymes in vitro, and at the same time abundantly transcribes the hydrophobin gene *ssg A* (146). St Leger et al (146) suggested that the SSGA hydrophobin (Figure 1) is involved in building the wall of the haustorium and could assist in hydrophobic attachment to the cuticular surface. Talbot et al (155) detected abundant transcription of the hydrophobin gene *MPG1* during infection, in planta, of rice plants with *Magnaporthe grisea* (Figure 1). *MPG1* mRNA was highly abundant very early in infection, concomitant with appressorium formation, while a second peak of

*MPG1* mRNA occurred during symptom development. They also performed a gene disruption and observed that the *Mpg1* mutants had reduced ability to cause disease symptoms that appeared to result from an impaired ability to undergo appressoria formation. Since appressorium formation is triggered in this case by a hydrophobic surface (58), the absence of a hydrophobin-mediated contact between the fungal wall and the inducing surface may suppress generation of a morphogenetic signal for appressorium formation. Similar to the effect of disruption of *rodA* in *A. nidulans* (147) and *eas* in *N. crassa* (10, 85), disruption of *MPG1* in *M. grisea* also caused the easily wettable phenotype of conidiospores (155). This suggests that the hydrophobin may also be important for dispersal of conidia of this phytopathogen. In addition, the suggestion was made (155) that the late expression of *MPG1* during disease development might be related to a phytotoxic effect of the hydrophobin, similar to that suggested for cerato-ulmin in Dutch elm disease.

Cerato-ulmin is produced extracellularly in large quantities by *Ophiostoma (Ceratocystis) ulmi,* and shares with the Sc3p hydrophobin the property of assembling at a water-air interface (122, 154). However, the cerato-ulmin aggregates are much less stable and dissociate readily in water and aqueous alcohol. Cerato-ulmin is also found on aerial structures of the fungus (150). The amino acid sequence of cerato-ulmin was determined (184) and a coding sequence subsequently synthesized and expressed in *E. coli* (12). The gene from *O. ulmi* has also been isolated recently (16). Cerato-ulmin shows homology to the other hydrophobins, particularly with respect to the spacing of the eight cysteine residues, but the hydropathy pattern around these residues clearly differs (Figure 1). Its presumed role as a wilt toxin is mainly based on the correlation found between production of cerato-ulmin and degree of virulence of the fungus (18).

The chestnut-blight fungus *Cryphonectria (Endothia) parasitica* produces large quantities of a similar protein, named cryparin (29). Like cerato-ulmin, it was collected from the culture medium on the surface of air bubbles (foam) and was soluble in aqueous alcohol. It was also present on aerial structures of the fungus. Hypovirulent strains containing cytoplasmically transmissible dsRNA contain much less cryparin, which suggests a role in disease development although no phytotoxic effects have yet been established (29). The cryparin gene was recently cloned and the deduced amino acid sequence found to be 50% homologous to that of cerato-ulmin (187). The hydropathy patterns are even more similar (Figure 1). During rapid growth in liquid cultures the cryparin mRNA accounts for 25% of the whole mRNA mass (187), which is an extreme example of the high abundance of hydrophobin mRNAs found in other fungi. Although the roles of both cerato-ulmin and cryparin as wilt toxins are under debate (29), they could both act by impairing water transport in xylem vessels by blocking bordered pits or by causing embolisms (160) by

coating and stabilising air bubbles. We found that an abundantly expressed gene identified in the nonpathogen *Trichoderma reesei* (T Nakari-Setälä & M Pentillä, unpublished data) also encodes a class II hydrophobin very similar to cerato-ulmin and cryparin. These proteins are thus not specific for phytopathogens.

Apart from their role in spore dispersal, adhesion to plant surfaces, and toxicity, hydrophobins may be involved in growth of the fungus in the plant, e.g. during formation of haustoria. Since hydrophobins are abundantly excreted as diffusible proteins, unless assembled on the fungal surface, they may also act as specific elicitors of the plant defense. Hydrophobicity of the fungal surface has also been suggested to play a major role in fungal infections in humans (63, 64). It would therefore be of great interest to examine the presence of hydrophobins in human-pathogenic fungi, for instance the opportunistic pathogens *Candida albicans* and *Aspergillus fumigatus*. Excreted soluble hydrophobins may be targets for immunological diagnosis of mycoses. If hydrophobins are essential for infection, they may become targets for drug development.



## CONCLUSIONS

Apical wall biogenesis is an activity of the polarized cytoplasm of fungal hyphae. The wall at the apex is initially plastic but, while falling behind the apex, gradually hardens due to crosslinking of the wall polymers. Variations in spatial distribution of wall synthesis is suggested to be responsible for the generation of different shapes, including those of infection structures. Turgor may assist in wall expansion but is certainly important for invasive growth of hyphae. Protein excretion also occurs primarily at hyphal apices, and the flow of wall material occurring during expansion may translocate proteins through the wall. Among these excreted proteins are the newly discovered hydrophobins, which can assemble at a hydrophillic-hydrophobic interface and are suggested to play important roles in morphogenesis and pathogenesis.

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