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Review article

Hydrophobins in Ectomycorrhizal Symbiosis: Hypothesis

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Abstract

In this review, are summarized data on the identification of hydrophobin genes in the ectomycorrhizal fungus *Pisolithus tinctorius*. Hydrophobins are involved in many developmental processes of saprotrophic and pathogenic fungi, and we propose a hypothesis for their functions during ectomycorrhizal symbiosis establishment. Hydrophobins, which are probably localized outside the cells, are likely to be involved in fungal cells attachment to the host surface and/or in hyphal aggregation around the root.

Keywords: Adhesion, basidiomycete, cysteine-rich polypeptide, host-microbe interaction, mycorrhizae, symbiosis

1. Introduction

Hydrophobins: the roots

Growth of fungi occurs mainly by hyphae which may branch to form a mycelium. This mycelium usually fixes or penetrates a substrate in order to either get nutrients or infect a host, and accomplish its sexual development.

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The adhesion onto a substrate clearly involves extramatrical molecules and cell wall structures. Carbohydrates and proteins are probably largely responsible for the physical forces required for adhesion. In the early 90's, new fungal proteins were discovered by Wessels' group, while searching for genes involved in fruit-body formation and emergent growth of the mycelium from the saprotrophic fungus *Schizophyllum commune* (for reviews, see Wessels et al., 1995; Wessels 1996; 1997). The polypeptides encoded by these genes (*SC1, SC3, SC4* and *SC6*) are small cysteine-rich hydrophobic proteins called hydrophobins. In the meantime, hydrophobin genes were discovered in many other filamentous fungi (among them *Aspergillus, Neurospora*) (de Vries et al., 1993), indicating a wide-spread distribution of these polypeptides in fungal species. General traits for these proteins have been recently proposed by Wessels (1997): hydrophobins are small proteins and their hydrophobicity varies from one member to an other. They are secreted (presence of a signal sequence) and have a conserved motif of eight cysteine residues. The homology at the amino-acid level between several sequences is very poor, even for different members of the same species. And finally, monomers of hydrophobins can assemble into an amphipathic layer at an hydrophilic-hydrophobic interface, to cover the external surface of an hypha or a spore by an hydrophobic material.

Role of hydrophobins in fungal development

The ubiquity of hydrophobins in the fungal phylum is illustrated by the diversity of function of these proteins in many developmental processes. The hydrophobin genes are differentially expressed in monokaryotic and dikaryotic hyphae, indicating a regulation by the mating type determinants (Schuren and Wessels, 1990). Furthermore, hydrophobins are abundant in fruit bodies and some members of hydrophobin genes are switched on during fruit body formation (Wessels et al., 1991a; De Groot et al., 1996). In asexual development, conidiophore differentiation is correlated with the expression of hydrophobin genes. Spore surfaces are known to often be decorated by rodlet structures which completely disappear when hydrophobin genes are disrupted: hydrophobins are thus the major component of the spore surface (Stringer et al., 1991; Bell-Pedersen et al., 1992; Lauter et al., 1992; Parta et al. ,1994; Thau et al., 1994; Bidochka et al., 1995; Stringer and Timberlake, 1995). Finally, hydrophobins were also described in pathogenic fungi where they are probably involved in the binding onto plant or animal host surfaces (St. Leger et al., 1992; Talbot et al., 1993).

The different roles of hydrophobins during hyphal morphogenesis can be explained in the light of many biochemical studies performed on the purified SC3p hydrophobin from *S. commune*. One hypothesis is that SC3p monomers of hydrophobins are excreted at the tip of hyphae growing in a liquid medium. When hyphae reach the air surface, SC3p monomers assemble into an insoluble hydrophobin monolayer responsible for the hydrophobicity of the hyphal surface (Wösten et al., 1994a). This assembly property derives from the amphipathic structure of SC3p: the protein can orientate its hydrophobic and hydrophilic parts towards hydrophobic or hydrophilic surfaces, respectively (Wösten et al., 1994b). This hydrophobic surface probably plays a role in spore dissemination and in binding of hyphae to any hydrophobic surface: either another hypha (to form a fruit body for instance) or a host surface (a plant or insect cuticle).

Fungal morphogenesis induced by ectomycorrhiza formation

Ectomycorrhizal fungi belong to the Basidiomycetes or Ascomycetes and share many characteristics in their biology with the fungi where hydrophobins were described: they are able to form emergent structures (fruit bodies), produce billions of hydrophobic spores and form aggregates in the soil (rhizomorphs). Their peculiarity is in their ability to colonize a root by adhering to its surface and by forming a fungal sheath – the ectomycorrhizal mantle – made of aggregated hyphae. This symbiotic structure – the ectomycorrhiza – culminates in the formation of an exchange zone between the two partners (the Hartig net) through the penetration of specialized hyphae between the root cells. This structure allows the transfer of nutrients from one partner to another (Smith and Read, 1996). Hyphae at the vicinity of a root switch their growth habit to develop a highly branched mycelium which contacts and anchors the root surface (Jacobs et al., 1989). The emission of a mucilaginous material rich in carbohydrates and proteins accompanies this colonization step (Lei et al., 1990). This colonization ends by the formation of a synenchyma tissue around the root, made of tightly aggregated hyphae. Thus, formation of ectomycorrhiza induces many changes in fungal cell morphology and is accompanied by host surface colonization and aggregation of hyphae.

Our current research is to characterize genes and proteins whose expression are regulated during ectomycorrhiza formation. Due to the importance of the cell wall both in the early step of contact and in the late stage of functioning (Hartig net), cell wall components were particularly examined (Tagu and Martin, 1996).

2. Hydrophobins in Ectomycorrhizas: The Facts

Characterization

Since hydrophobins seem to be involved in adhesion and surface host recognition, we were interested in looking at hydrophobin expression in ectomycorrhizal species. Searching for hydrophobin cDNAs in a library is very difficult since the similarity in amino acid sequences is low. However, Parta et al. (1994) and Thau et al. (1994) were able to clone the hydrophobin *RodA* gene from *Aspergillus fumigatus* by using the *RodA* gene sequence from the related species *A. nidulans*. We tried several times to clone hydrophobin cDNAs from the ectomycorrhizal fungus *Pisolithus tinctorius* by using either the *SC3* gene from *S. commune* or degenerated *SC3* primers (designed by Sietsma and Wessels, unpublished): we were not able to succeed in these trials. In the meantime, we were involved in the characterization of abundant transcripts in ectomycorrhizas by cataloguing Expressed Sequence Tags (EST) from *Eucalyptus globulus-Pisolithus tinctorius* symbiotic tissues (Tagu and Martin, 1995). In a

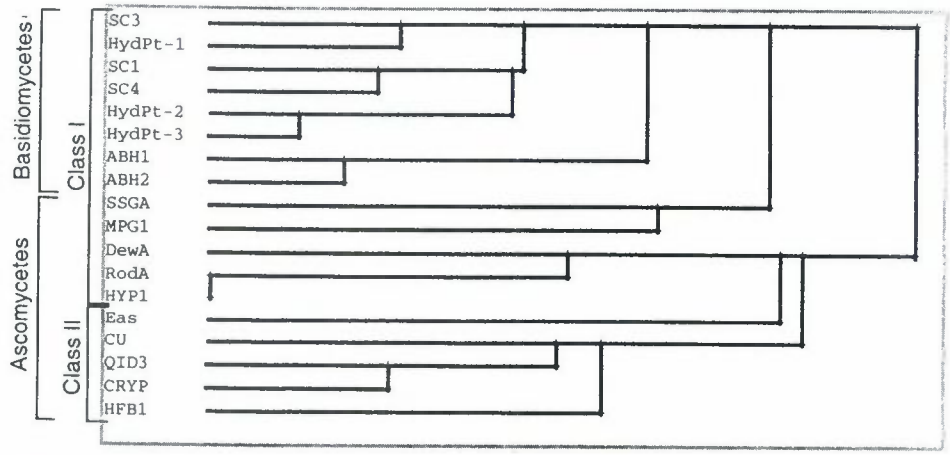

Figure 1. Dendrogram of similarities between aligned hydrophobins. Alignments were computed with MultAlin programme (Corpet, 1988) on-line at the ProDom site (http://protein.toulouse.inra.fr/prodom.html). The dendrogram presented in this figure differs from the one proposed by Wessels (1997) since we used for comparison the complete sequences, including the putative signal sequences. References: Schuren and Wessels (1990); Stringer et al. (1991); Bell-Pedersen et al. (1992); Lauter et al. (1992); St Leger et al. (1992); Talbot et al. (1993); Bowden et al. (1994); Lora et al. (1994); Parta et al. (1994); Thau et al. (1994); Zhang et al. (1994); Stringer and Timberlake (1995); de Groot et al. (1996); Nakari-Setälä et al. (1996); Tagu et al. (1996).

pilot experiment, we identified three EST presenting similarity with *S. commune* hydrophobins. Further sequencing of 300 new EST (Voiblet and Martin, unpublished) revealed the presence of numerous hydrophobins cDNAs representing nearly 5% of the EST. This indicates that hydrophobins might represent one of the major proteins synthesized in *Pisolithus* ectomycorrhizas.

Three different hydrophobin cDNAs were thus characterized from *P. tinctorius*, and called *hydPt-1*, *hydPt-2* (Tagu et al., 1996), and *hydPt-3* (Tagu, Voiblet and Martin, unpublished). They share between 35% and 51% similarity. The polypeptide sequences deduced from the three nucleic acid sequences present the same distribution of hydrophobic domains (Tagu et al., 1996) and the 8 cysteine residues at the conserved positions. They are small neutral or acidic polypeptides (Table 1) and present a high probability of having a signal peptide. Thus, these proteins are probably cell wall located or excreted. A dendrogramme of similarities indicates that *Pisolithus* HydPt belong to the cluster of hydrophobins from Basidiomycetes species (Fig. 1; Wessels, 1997).

Table 1. Biophysical parameters of *P. tinctorius* hydrophobins. The data were obtained from the deduced amino acid sequences of the three cDNAs *hydPt-1*, *hydPt-2* and *hydPt-3*. Values were calculated by the ProtParam (http://expasy.hcuge.ch/sprot/protparam.html) and the PSORT (http://psort.nibb.ac.jp) tools available on-line in the ExPASy Web site (Swiss Prot Center, http://expasy.hcuge.ch).

	HydPt-1	HydPt-2	HydPt-3
Number of amino acids	140	117	108
Presence of signal peptide (probability)	0.70	0.82	0.69
Length of signal peptide (amino acids)	18	21	17
Molecular weight of mature protein (kDa)	12.3	9.7	9.4
Theorical pI of mature protein	6.94	5.91	4.14

RNA expression

The *hydPt-1*, *hydPt-2* and *hydPt-3* mRNA are abundant in free living hyphae: their detection in a standard Northern blots only requires few hours of exposures (Tagu et al., 1996; Tagu, Voiblet, Martin unpublished). They probably represent the major transcripts of the *P. tinctorius* mycelium developing aerial hyphae since these experiments were done by harvesting the mycelium growing on top of a agar medium covered by a Cellophane membrane. However, *hydPt-1* and *hydPt-2* mRNA extracted from the bottom of a colony

floating on a liquid medium are rare. Thus, *hydPt-1* and *hydPt-2* RNA accumulation is dependent on the growth habit of the mycelium. Hydrophobic (aerial) hyphae are richer in these transcripts than hydrophobic (immerged) hyphae. Data for the more recently isolated *hydPt-3* cDNA are not yet available.

When the mycelium is challenging the eucalypt root, the level of *hydPt-1*, *hydPt-2* and *hydPt-3* mRNA is increased from 3 to 4 times, at the early stage of colonization corresponding to the binding of the mycelium to the root surface (Tagu and Martin, 1996; Tagu et al., 1996). This indicates that *Pisolithus-Eucalyptus* ectomycorrhiza formation is accompanied by an up-regulation of hydrophobin genes, leading to an overproduction of the corresponding polypeptides.

3. Hydrophobins in Ectomycorrhizas: Hypothesis

The data obtained in *P. tinctorius-E. globulus* ectomycorrhizas suggest that a regulation of hydrophobin gene expression occurs during symbiotic structure development and that the hydrophobin polypeptides play a role in adhesion to the root surface and/or in binding hyphae together. However, our results are still scarce and need further evidences to conclude on the function of hydrophobins in ectomycorrhiza symbiosis. But, by analogy with what is known for saprotrophic and pathogenic fungi, our hypothesis might be proposed (Wessels 1996; 1997).

Regulation of gene expression

Hydrophobins are abundantly synthesized in different structures namely, fruit bodies, conidiophores and conidiospores. They are thus regulated by several morphogenetic processes (Table 2) and are probably under a tight regulation by master genes involved in fruit body or conidiophore development (Wessels et al., 1991a). They are also dependent on the ploidy level since *S. commune* monokaryotic or dikaryotic mycelia do not express the same hydrophobin genes. Furthermore, the aerial hyphae, probably at the initiation of fruit body primordium differentiation, expressed also their own set of hydrophobins (Wessels et al., 1991b). *HydPt-1*, *hydPt-2* and *hydPt-3* cDNAs were characterized from dikaryotic aerial hyphae of *P. tinctorius*. Regulation of the expression by mating type genes or ploidy level is difficult to test since the germination yield of *P. tinctorius* spores is very low and the regeneration of the primary mycelium is too poor in order to isolate monokaryotic hyphae *in vitro*.

Table 2. Examples of regulation of the steady-state level of hydrophobin transcripts and polypeptides. *thn*: recessive mutation which suppresses the formation of aerial hyphae in the monokaryon and the formation of fruit-bodies in the dikaryon (Wessels et al., 1991b). *fbf*: recessive mutation which does not change the phenotype in the monokaryon but which suppresses the formation of fruit bodies and induces the development of aerial hyphae in the dikaryon (Wessels et al., 1991a). *npr1* and *npr2*: mutations that hinder utilization of nitrogene sources of *Magnaporthe grisea* (Lau and Hamer, 1996). *aba A*, *brlA* and *wetA*: regulatory genes of conidiogenesis (Stringer et al., 1991; Stringer and Timberlake, 1995). *fl* and *bd;frq*: mutations which provoke a deregulation of circadian clock in *Neurospora crassa*. *wc-1* and *wc-2*: genes encoding proteins which act in the light-activated signal transduction pathway in *Neurospora crassa* (Arpaia et al., 1993). db-RNA: double stranded RNA in infectious hyphae (Zhang et al., 1994). nd: non determined. Other references: Bell-Pedersen et al. (1992); Lauter et al. (1992); St. Leger et al. (1992); Talbot et al. (1993); Bowden et al. (1994); Parta et al. (1994); Thau et al. (1994); De Groot et al. (1996); Nakari-Setälä et al. (1996); Tagu et al. (1996).

Gene	Organism	Tissue where accumulated	Regulatory genes	Developmental regulation
sc1, sc4, sc6	*Schizophyllum commune*	Fruit body	*thn, fbf*	Mating type, fruit body, dikaryotic mycelium
sc3	*Schizophyllum commune*	Aerial hyphae	*thn*	Mating type, mono and dikaryotic mycelium
mpg1	*Magnaporthe grisea*	Infection structure (appressoria)	*npr1, npr2*	Conidiogenesis, carbon and nitrogen deprivation
rodA (hyp1)	*Aspergillus nidulans, A. fumigatus*	Conidiospore, conidiophore	*brlA, abaA*	Conidiogenesis
dewA	*Aspergillus nidulans*	Conidiospore	*wetA*	Conidiogenesis
ccg-2 (eas)	*Neurospora crassa*	Conidiospore	*fl, bd;frq, wc-1, wc-2*	Conidiogenesis, circadian clock, blue light
ssgA	*Metarhizum anisopliae*	Infection structure (appressoria)	nd	Carbon and nitrogen deprivation
hypA	*Agaricus bisporus*	Fruit body	nd	Fruit body formation
cu	*Ophiostoma ulmi*	Infection structure	nd	nd
crp	*Cryphonectria parasitica*	Infection structure	db-RNA	nd
hfb1	*Trichoderma reesei*	Hyphae	nd	Carbon induction
hydPt-1, hydPt-2, hydPt-3	*Pisolithus tinctorius*	Infection structure (mycorrhiza)	nd	nd

The data obtained from pathogenic fungi indicate that nutrient deprivation can trigger the expression of hydrophobin genes (St. Leger et al., 1992; Talbot et al., 1993). The pathogenic mycelium reaching the host surface enters a zone depleted in nutrients: the subsequent induction of hydrophobin would help the mycelium to grow towards the host surface. It is tempting to make a parallel with the expression of some avirulent genes from pathogenic fungi which are also induced by a nitrogen deprivation (Van den Ackerveken et al., 1994). This could be a general and non specific way for a fungal mycelium to sense the presence of a putative host surface before the colonization step. A mycorrhizal mycelium growing around a root system enters a zone poor in nutrients (Smith and Read, 1997); this could enhance the hydrophobin production through gene activation early in the process of colonization. Carbon source was also shown to be up-regulating the *hfb1* hydrophobin gene from *Trichoderma reesei* (Nakari-Setälä et al., 1996): the proximity of root exudates rich in carbon nutrients could also increase the synthesis of hydrophobins in ectomycorrhizal fungi.

These observations strengthen the hypothesis proposed by Templeton et al. (1994) that avirulent proteins and hydrophobins might be evolutionary related. These proteins are both small, excreted and rich in cysteines distributed in a similar way. However, avirulent proteins usually confer a high host specificity, whereas hydrophobins are ubiquitous (found in pathogenic, saprotrophic and symbiotic mycelia) and their involvement in specificity remains to be proved. Directed mutagenesis of hydrophobins or avirulent coding sequences and disruption of the native genes are necessary in order to conclude on their similarity of function.

The mechanism of perception of hydrophobins by the host surface is totally unknown: are they internalized into the host cells as it is proposed for some bacterial avirulent proteins (Lamb, 1996)? Do the hydrophobins have any effect on the physiology of root cells? Are they able to modify ion fluxes or gene expression as elicitins from *Phytophthora cryptogea* – another cysteine rich fungal protein – do on tobacco cells (Viard et al., 1994; Suty et al., 1995)?

Finally, the hydrophobin cryparin from the pathogenic fungus *Cryphonectria parasitica* is regulated by the presence of a hypovirulent double-stranded RNA (Zhang et al., 1994). The presence of such RNA should be checked in several ectomycorrhizal fungi as possible regulators of aggressiveness.

Function of the hydrophobin polypeptides

Hydrophobins are involved in adhesion and fixation of hyphae onto a host surface. These two phenomena take place during ectomycorrhiza development (i.e. mantle formation) and hydrophobins could be involved in one or both

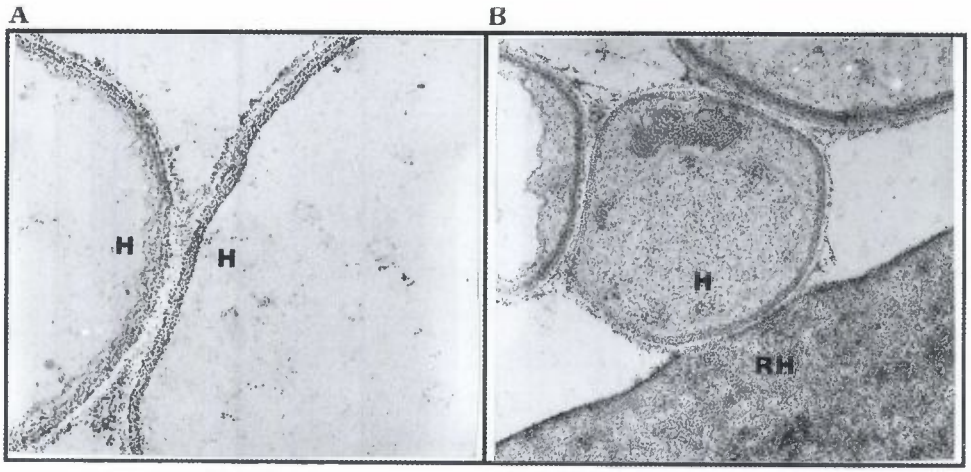

Figure 2. Early steps of root colonization. Fig. 2A shows that binding between hyphae is performed by the outer layer of the hyphal wall. The same layer also binds hyphae to root hairs (Fig. 2B) and probably to the root cell walls, although this was not demonstrated so far. The material was pre-fixed in gluteraldehyde-formalin according to Karnovsky (1965) in phosphate buffer, post-fixed in iron-sulfate, dehydrated by aceton and embedded in ERL according to Spurr (1969). Sections on gold grids were treated by 1% periodic acid for 45 min, washed, floated on silver methenamin solution for 90 min and washed in 10% sodium thiosulphate for 1 h. H: hyphae. RH: root hair. Magnifications: 47,500× in A and 28,500× in B.

processes. At least three different hydrophobin genes are expressed in *P. tinctorius* ectomycorrhiza, and each could have a specific function. Localization of these polypeptides in ectomycorrhizal tissues could partially answer to this question. However, the only way to univocally study the different roles of hydrophobins would be the inactivation of the corresponding genes by homologous recombination (genetic transformation of *P. tinctorius* has not been successful yet).

Following the hypothesis proposed by Wösten et al. (1994b), it is easy to imagine that *P. tinctorius* hydrophobins could form a hydrophobic monolayer at the surface of the hyphae responsible for the binding and the aggregation of hyphae together in the ectomycorrhizal mantle (Tagu and Martin, 1996). The binding of hyphae onto the root surface is less evident to understand since root surfaces are usually described as hydrophilic. But this surface could be heterogenous and scarce hydrophobic microdomains could be present. For instance, Kottke (1997) indicated the presence in some cases of an envelope

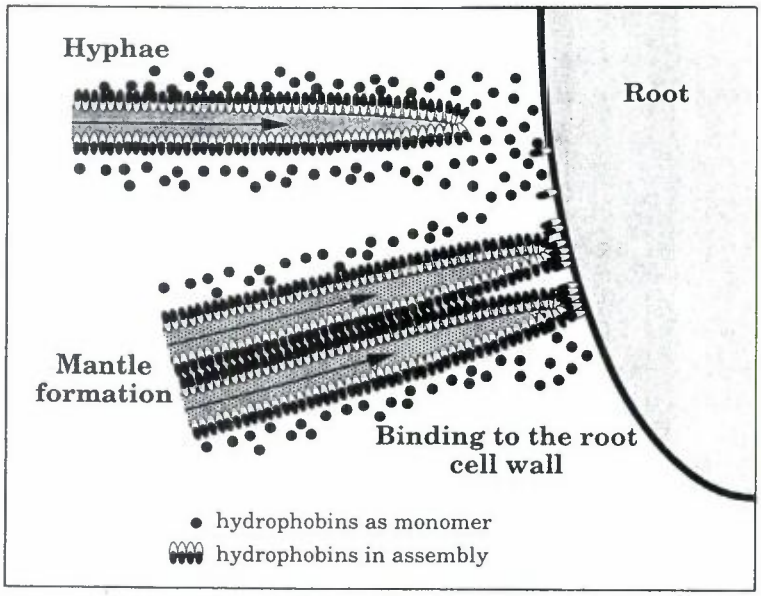

Figure 3. Model for the attachment of hyphae to hydrophilic root surfaces. Inspired by Wessels (1996). *Pisotihus tinctorius* hyphae, probably covered by an hydrophobin layer, might secrete monomers of hydrophobins in the medium. These monomers, by contacting the root surface, self assemble and direct their hydrophobic domains towards the external medium. Colonizing hyphae could then bind to these hydrophobic layers over the root cell wall.

around *Picea abies* roots which could explain a local hydrophobicity of the root surface. Piché et al. (1983) observed similar binding of *P. tinctorius* to root cap cells of *Pinus strobus*. The identification of such an envelop around eucalypt root (the host of *P. tinctorius* in our experiments) was less evident (Fig. 2B) but hyphae are still able to bind the root surface.

By adopting a model proposed by Wessels (1996), one can explain the binding of hyphae to hydrophilic surface by hydrophobins (Fig. 3). One set of hydrophobins could be excreted by the hyphae into mucilage and then bind the hydrophilic host surface and direct their hydrophobic domains towards the external medium. Then, hydrophobic hyphae, covered also by another set of hydrophobins, could bind to this hydrophobic host surface and allow the colonization stage. Moreover, as described by Ashford et al. (1988) for *Pisonia* mycorrhiza, there is an important decrease in apoplastic permeability as the fungal sheath differentiates and these changes could be the result of the secretion of extracellular material by the fungus, like hydrophobins. In any

case, our results are not enough documented and further studies on hydrophobin polypeptides from ectomycorrhizal fungi are needed.

4. Conclusions: Other Symbioses

Hydrophobins are probably involved in adhesion and fixation to the host surface in ectomycorrhizal symbiosis but the final demonstration remains to be done. These proteins are probably not the only actors of adhesion and likely correspond to parts from a whole made of other cell wall components as polysaccharides, mannoproteins ($SRAP_{30-32}$ polypeptides containing cell adhesion motif: Laurent, Tagu and Martin unpublished; Martin et al., 1995; Tagu and Martin, 1996) or lectins (Giollant et al., 1993).

P. tinctorius was the first symbiotic fungus from which hydrophobin genes were identified. However, recently, hydrophobin polypeptides from a lichen fungus were purified (R. Honegger, personal communication) demonstrating that these proteins are probably involved in many fungal symbioses. Hydrophobins have not been described yet in endomycorrhizal fungi but are probably present. They might be involved in appressoria formation as for pathogenic fungi. Due to the poor similarity between gene sequences, the use of heterologous probes is probably not possible to clone the hydrophobins from endomycorrhizal fungi. A biochemical approach should be more appropriate. However the difficulty to grow endomycorrhizal mycelia in pure culture renders the task even harder.

Other hydrophobin-like proteins may exist in fungi as described for the fungal pathogen *Ustilago maydis* (Wösten et al., 1996) and the process of adhesion of symbiotic mycorrhizal hyphae may involve many different polypeptides similar or not to hydrophobins.

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