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**Comparative genomics and expression levels of hydrophobins from eight mycorrhizal genomes**

[Short title: Hydrophobins in mycorrhizal genomes]

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***Abstract:*** Hydrophobins are small secreted proteins that are present as several gene copies in most fungal genomes. Their properties are now well understood: they are amphiphilic and assemble at hydrophilic/hydrophobic interfaces. However, their physiological functions remain largely unexplored, especially within mycorrhizal fungi. In this study, we identified hydrophobin genes and analyzed their distribution in eight mycorrhizal genomes. We then measured their expression levels in three different biological conditions (mycorrhizal tissue *vs* free-living mycelium; organic *vs* mineral growth medium; and aerial *vs* submerged growth). Results confirmed that the size of the hydrophobin repertoire increased in the terminal orders of the fungal evolutionary tree. Reconciliation analysis predicted that in 41% of the cases, hydrophobins evolved from duplication events. Whatever the treatment and the fungal species, the pattern of expression of hydrophobins followed a reciprocal function, with one gene much more expressed than others from the same repertoire. These most expressed hydrophobin genes were also among the most expressed of the whole genome, which suggests that they play a role as structural proteins. The fine-tuning of the expression of hydrophobin genes in each condition appeared complex because it differed considerably between species, in a way that could not be explained by simple ecological traits. Hydrophobin gene regulation in mycorrhizal tissue as compared with free-living mycelium, however, was significantly associated with a calculated high exposure of hydrophilic residues.

***Key words:*** Comparative genomics; Hydrophobins; Mycorrhizas; Small secreted proteins

**Introduction**

Hydrophobins are small proteins secreted only by fungi. They are characterized by three peculiar features: a short sequence (less than 200 amino acids), a secretion signal, and a recurrent pattern containing eight cysteine residues (Wessels et al., 1991). These features are responsible for their unique three-dimensional folding, which keeps the hydrophobic residues in an exposed patch, making them amphiphilic. Moreover, when their concentration reaches a certain threshold, the β-sheets of model hydrophobins have the property of stacking together, leading them to assemble in linear superstructures, called rodlets (de Vocht et al., 1998). These rodlets can be observed with atomic force microscopy (Wösten & de Vocht, 2000), and are responsible for the hydrophobicity of fungal cell walls. Hydrophobins are divided in two types, based on their properties: Type I and II. The Type I peptides are produced by both Asco- and Basidiomycetes, have higher sequence variability and assemble in more stable superstructures than the Type II peptides, that only are produced by Ascomycetes (Kershaw & Talbot, 1998). Thanks to their peculiar properties, hydrophobins are involved in at least two physiological processes: adhesion to hydrophobic surfaces (Wösten et al, 1994) and formation of aerial hyphae (Wessels et al., 1991). These processes are themselves required for several biological functions, the two best described ones being attachment of pathogen spores to the waxy cuticle of leaves and other plant tissues (Whiteford & Spanu, 2002; Linder et al., 2005) and elaboration of fruiting bodies (de Groot et al., 1996). But these characteristic properties of hydrophobins also could be involved in other biological functions. First, they may contribute to the initial establishment of the mycorrhizal symbiosis. Some hydrophobins indeed were found upregulated during the establishment of the symbiosis by *Pisolithus tinctorius* (Tagu et al., 1998) and *Laccaria bicolor* (Martin et al., 2008; Plett et al., 2012). Moreover, Mankel et al (2002) found that one hydrophobin

produced by the ectomycorrhizal (ECM) fungus *Tricholoma terreum* was found in both the mantle and the Hartig net of the mycorrhiza, but was much less expressed and abundant in the Hartig net for a non-compatible host. How hydrophobins are involved in the interaction with the host still is unclear, however, but results obtained so far suggests that they could facilitate the adhesion of the mycelium to the plant root (Raudaskoski & Kothe, 2015). This hypothesis implies that endomycorrhizas would rely much less on hydrophobins for the establishment of their symbiosis, because attachment is only locally and transiently necessary for the penetration of plant cells by their hyphae. Second, hydrophobins may act as biosurfactants, thereby assisting in organic matter adhesion and subsequent degradation. Biosurfactants increase the bioavailability of hydrophobic molecules (Ron & Rosenberg, 2001), such as most of the soil organic polymers which are potentially degraded by ectomycorrhizal fungi foraging for N (cellulose, hemicellulose, lignins, chitins, soil proteins; Shah et al., 2016). Third, hydrophobins can contribute to the growth of the fungus at the surface of a liquid, by lowering water surface tension, allowing hyphae eventually to escape from water to the air (Wösten et al., 1999). These aerial, hydrophobic structures may be of course involved in the formation of sporocarps, but also in the development of the hydrophobic mantle typical of many ectomycorrhizas (Agerer, 2001) or simply the passage of external hyphae through aerial spaces in upper forest soil layers. Endomycorrhizal fungi produce only primitive fruiting bodies, and do not differentiate a large part of their biomass into a mantle as ectomycorrhizal fungi do. Therefore, theorically they require much less of the functions potentially provided by hydrophobins than their ectomycorrhizal counterparts, even though they may still differentiate some of their mycelium to hydrophobic structures such as rhizomorphs.

Hydrophobins already have been investigated in the genomes of some model saprophytic and pathogenic fungal species. It appears that the size of the hydrophobin family differs considerably among species (from 3 in *Postia placenta* to 40 in *Trametes versicolor*), but that most of the fungi have at least several hydrophobin genes (Mgbeahuruike et al., 2013). This suggests that these proteins potentially are involved in several distinct biological functions.

Several recent reports on model hydrophobins show that their functional properties depend on their structural attributes. The N-terminal part of the sequence, for example, appears to determine the wettability of the hydrophilic side of the protein (Wösten & de Vocht, 2000). Lienemann et al. (2013) showed that the nature and position of the charged amino acids in the sequence of the protein influenced its ability to bind to surfaces and to form rodlets. The rodlet formation itself depends on the ability of the protein to fold into β-sheet structures which can stack over each other into an amyloid structure (Mc Indoe et al., 2012); this three dimensional folding also results in the hydrophobic amino acids being grouped in the same region of the rodlet (Kwan et al., 2006). The amino acids between the cysteines 3 and 4 make a large disordered loop, that does not contribute to the formation of rodlets (at least in the hydrophobin I model EAS), but will make up an important surface on its exposed part (Kwan et al., 2006); hence, their charge and hydrophobicity will influence significantly the functional properties of the protein assembly.

Our aim was to gain insights about the potential roles of mycorrhizal hydrophobins by comparing the size, sequence, expression levels and calculated structural properties of hydrophobin genes in mycorrhizal genomes in different conditions. For this purpose, hydrophobin genes and their structure were identified in the genomes of eight mycorrhizal fungi sequenced by the Mycorrhizal Genomics Initiative (Kohler et al., 2015). We subsequently analyzed the clustering of the hydrophobin genes in the genomes, compared their sequences,

calculated structural properties, and measured their expression levels in three different conditions: free living mycelium vs. ectomycorrhizal tissue (to identify hydrophobins potentially involved in mycorrhiza formation); mycelium growing on mineral medium vs. soil organic matter extract (hydrophobins potentially affecting biosurfactant activity); and mycelium growing submerged vs. aerial (hydrophobins potentially affecting formation of aerial hyphae).

**Material & methods**

*Identification of hydrophobin genes*

We identified hydrophobin genes within each of eight sequenced mycorrizal genomes of ectomycorrhizal fungi (ECM) *Suillus luteus V1.0, Paxillus involutus V1.0, Laccaria bicolor V2.0, Hebeloma cylindrosporum V2.0, Piloderma croceum V1.0*, the orchid mycorrhizal fungi (ORM) *Tulasnella calospora V1.0, Serendipita vermifera V1.0* and the ericoid mycorrhizal fungus (ERM) *Oidiodendron maius V1.0*) following a protocol inspired by Plett et al., 2012. Briefly, we first retrieved a list of 96 "reference" hydrophobins from the genomes of *Coprinopsis cinerea, Agaricus bisporus, Agrocybe aegerita, Dictyonema glabratum, Pleurotus ostreatus, Pisolithus tinctorius, Schizophyllum commune* and *Neurospora crassa* (20, 20, 1, 3, 7, 3, 32 and 10 protein sequences, respectively). For each of the eight queried genomes, we used the following procedure: 1) each of these "reference" hydrophobin sequences were blasted against each of the genomes (filtered gene models database, BlastP and tBlastn, threshold e value=$10^{-5}$); 2) all the proteins predicted to contain the InterPro annotation corresponding to Type I hydrophobins (IPR001338) were retrieved; and 3) we recovered all protein sequences bearing the hydrophobin Type I (C-$X_{5–8}$-C-C-$X_{17–39}$-C-$X_{8–23}$-C-$X_{5–6}$-C-C-$X_{6–18}$-C-$X_{2–13}$) or Type II (C-$X_{9–10}$-CC-$X_{11}$-C-$X_{16}$-C-$X_{8–9}$-C-C-$X_{10}$-C-$X_{6–7}$) signature sequences, using a homemade Perl script. Then, all the sequences retrieved through at least one of the three methods were re-blasted (tBlastn, threshold evalue=$10^{-5}$) against each genome. We ended up with a list of 6 to 38 potential hydrophobin sequences, depending on each query genome (193 in total). We then filtered-out putative false positives, which we defined as sequences that were retrieved only through signature search and /or re-Blast (because none of these showed any blast hit with a known hydrophobin in the NCBI nr database: Blastp, threshold e-value=$10^{-3}$). As a result, we ended up with a list of 82 potential hydrophobin genes. No hydrophobin gene could be found in the *T. calospora* genome. To compare sequences of the mycorrhizal hydrophobins we added twenty (20) "model" hydrophobins the roles of which previously have been described in other fungi (saprotrophic or biotrophic) or of which expression levels previously have been measured (Table 1). All sequences were retrieved from the NCBI database.

*Table 1. Name and characteristics of the twenty "model" hydrophobins used in this study. The proteins are sorted by species (first column). Attributes of each hydrophobin summarizes what is known about their properties and functions. References supporting the attributes are shown.*

| Species of origin | Gene name | Attributes | Reference |
|---|---|---|---|
| *Schizophyllum commune* | SC3 | Form rodlets. Surfactant action. | Lugones et al., 1998 |
| | SC4 | Form rodlets. Surfactant action. Line air channels in fruting bodies | Lugones et al., 1999 |
| *Agaricus bisporus* | Ab ABH1 | Form rodlets. Surfactant action. Line air channels in fruting bodies | Lugones et al., 1999 |
| | Ab ABH3 | Formation of hydrophobic aerial hyphae and attachment of hyphae to hydrophobic surfaces; hydrophobic peel of the fruiting body. | Lugones et al., 1998 |
| *Pleurotus ostreatus* | Po POH1 | Expressed only in fruiting bodies, not in vegetatve mycelium | |
| | Po POH2 | Form rodlets, secreted in the medium, and found in aerial hyphae | Asgeirdottir et al., 1998 |
| | Po POH3 | Form rodlets, secreted in the medium, and found in aerial hyphae | |
| | Po vmh1 | Found mostly in vegetative mycelium | |
| | Po vmh2 | Found mostly in vegetative mycelium | Penas et al., 2002 |
| | Po vmh3 | Found mostly in fruiting bodies | |
| *Flammulina velutipes* | Fv fvh1 | Expressed mostly during fruiting body initiation | Ando et al., 2001 |
| *Pholiota nameko* | Pn pnh1 | Expressed in situation of phosphate starvation | |
| | Pn pnh2 | Expressed in situation of phosphate starvation | Tasaki et al., 2004 |
| | Pn pnh3 | Expressed in situation of phosphate starvation | |
| *Heterobasidion annosum* | Ha hah1 | Expressed in aerial hyphae, but not in submerged condition nor in plant infection stage | Karlsson et al., 2007 |
| | Ha hah2 | Expressed in aerial hyphae, but not in submerged condition nor in plant infection stage | |
| *Pisolithus tinctorius* | Pt HYdPt1 | Found in aerial hyphae and at early stages of mycorrhization | Tagu et al., 1996 |
| | Pt HYdPt2 | Found in aerial hyphae and at early stages of mycorrhization | |
| *Tricholoma terreum* | Tt Hyd1 | Found in aerial mycelium of the hyphal mantle and Hartig net, but only in a well compatible host | Mankel et al., 2002 |
| *Trichoderma reesei* | HFBII | Model type II hydrophobin, used in this paper as an outgroup | Whiteford & Spanu, 2002 |

*Biological conditions tested*

We tested six different biological conditions (because of different growth requirements among species, conditions 1 and 2 were prepared according to species-specific protocols; see Supplementary material for more details): 1. Free-living mycelium (referred as FLM in the figures). The fungi were grown aseptically on agar medium. All eight mycorrhizal fungi were investigated for this condition. / 2. Symbiotic tissue (SYM). The fungi were inoculated to a host plant in microcosms and mycorrhizal root tips were collected. All eight mycorrhizal fungi were investigated for this condition. / 3. Mineral medium. Five fungi (*S. luteus*, *P. involutus*, *L. bicolor*, *H. cylindrosporum*, *P. croceum*) were grown on top of a liquid mineral medium (we used Modified Melin-Norkrans medium, referred to as MMN throughout the manuscript. Composition: 2.5 g l–1 glucose, 500 mg l–1 $KH_2PO_4$, 200 mg l–1 $NH_4Cl$, 150 mg l–1 $MgSO_4 \cdot 7H_2O$, 25 mg l–1 NaCl, 50 mg l–1 $CaCl_2$, 12 mg l–1 $FeCl_3 \cdot 6H_2O$ and 1 mg l–1 thiamine-HCl; pH 4.0) in a glass beads system (see Shah et al., 2013 for a description of the glass bead system). / 4. Organic medium (OM). Five fungi (*S. luteus*, *P. involutus*, *L. bicolor*, *H. cylindrosporum*, *P. croceum*) were grown on top of a sterile liquid forest soil extract in a glass bead system. / 5. Submerged growth condition (SUB). The ECM fungus *S. luteus* was grown submerged in liquid MMN medium. / 6. Aerial growth condition (AER). The ECM fungus *S. luteus* was grown at the surface of liquid MMN medium.

Each condition consisted of at least three biological replicates. For each sample, total RNA was isolated, converted into cDNA and analyzed by qPCR (submerged vs. aerial conditions) or sequenced by RNA-seq (Illumina, all other conditions). The transcript abundances were normalized, and significantly differentially expressed genes were identified by comparing expression ratios in pairwise comparisons of conditions: 1 and 2 (effect of mycorrhizal status), 3 and 4 (effect of an organic substrate) and 5 and 6 (effect of the type of growth of the mycelium) (see Supplementary material for the extended Material and Methods).

*Similarity tree*

Because hydrophobins display substantial sequence variation, apart from the conserved canonical motif of 8 cysteine residues, we compared three different approaches to align all the hydrophobin sequences identified in the mycorrhizal genomes, together with the saprophytic ones and the "model" hydrophobins (hence 102 hydrophobins in total): i) We performed multiple alignments on the full-length protein sequences, using four different algorithms and by manipulating several parameters. In a preliminary attempt, we compared MAFFT (Gap opening penalty -1/-2/-3), MUSCLE (UPGMB/NJ, Gap open -2.9/-10/-15; Gap extend -0.01/-1/-5), and CLUSTALW (Gap opening penalty 0.2/0.5/1/10; Gap extension penalty 0.1/0.2/1). MUSCLE and CLUSTALW were run on MEGA7. ii) We used a targeted approach by performing multiple alignment of the hydrophobin PFAM domain only (PF01185, retrieved by sequence search on the PFAM database), using the same algorithms and parameters as above. The best alignment of the cysteine residues was obtained with CLUSTALW (Gap opening 0.5, Gap extension 0.2). iii) We performed a multiple structural alignment of 3D protein models. This approach was justified by hydrophobin surface properties arising from their tertiary (or even quaternary) structure, rather than from their primary structure *per se*. Therefore, hydrophobins performing the same functional role could have very different primary structures but similar 3D foldings. We used several *de novo*-3D prediction algorithms on EAS, an hydrophobin whose 3D structure has been deciphered experimentally (EAS: Mc Indoe et al., 2012). These algorithms were: PHYRE2 (intensive mode) (Kelley & Sternberg, 2009), QUARK (Xu & Zhang, 2013) and RAPTORX (Källberg et al., 2012); 3D models were formatted into ".pdb" files and visualized using Jmol. RAPTORX provided a 3D model that best corresponds to the real 3D structure of EAS described by Mc Indoe et al. (2012), and was therefore chosen to predict the 3D structure of all 82 hydrophobins plus SC3 from *S. commune*. The latter was used further as a reference for the alignment, because it is the best characterized 3D structure of a Type 1 hydrophobin from a basidiomycete (De Vocht et al., 1998). An alignment then was performed using MUSTANG (Konagurthu et al., 2006). Finally, we compared how the three approaches (full-length multiple alignment, PFAM multiple alignment, 3D structural alignment) performed to align the hydrophobin sequences. Alignment of the PFAM region only in CLUSTALW proved to be best consistently to align the cysteine pattern (Table S1). The alignments based on 3D structure comprised 91% (+/- 5% in standard deviation) gaps, and were therefore not analyzed further. Either the incomplete knowledge of hydrophobin structures and their variability, or that we used EAS, a protein produced by an Ascomycete, to infer 3D structure of mostly Basidiomycete proteins, could explain those poor alignment results. Nevertheless, the EAS protein had the best resolved 3D structure by far, and also was well studied regarding the links between structure and function (Macindoe et al., 2012) which motivated our choice for this hydrophobin as a 3D reference model.

Phylogenetic trees were built on these alignments using a maximum likelihood algorithm in RAXML (Stamatakis et al., 2008). Trees then were displayed using MEGA7 (Tamura et al., 2007) and Evolview (He et al., 2016).

We analyzed possible gene duplication of the best hydrophobin tree by topological reconciliation analysis in Notung 2.8 (Stolzer et al., 2012); the species tree used for reconciliation was a neighbour-joining tree based on the ITS sequence of each species retrieved from the NCBI nucleotide database (internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence).

*Prediction of individual hydrophobin properties*

As mentioned in the Introduction, several structural parameters appear to have an important role in the function of the protein: i) the number of the O-glycosylation sites in the N-terminal part of the sequence, which indicates the "wettability" of the N-terminal part of the sequence (Wösten & de Vocht, 2000); ii) the nature of exposed amino acids (acidic, basic, aromatic, hydrophobic or hydrophilic: Lienemann et al., 2013); iii) the number of β-sheets and α-helices (Mc Indoe et al., 2012); iv) the hydrophobicity of the protein (whole protein, and of the loops 2 and 3 in particular: see Kwan et al., 2006 and Wessels, 2000), and v) their ability to aggregate in amyloid fibrils (MacKay et al., 2001). Therefore, we computed 14 parameters for each identified hydrophobin sequence: 1: The proportion of S and T at the N-terminal part of the protein (before the first CC)-a proxy for the proportion of O-glycosylation sites / 2-3-4-5-6: The exposition of acidic, basic, hydrophilic, hydrophobic and aromatic residues in the protein sequence (predicted using NetSurfP ver. 1.1: Petersen et al., 2009) / 7-8: The number of β-sheets and α-helices (predicted using the ZAGG calculator: Tartaglia & Vendruscolo, 2008) / 9-10-11: The GRAVY score (hydrophobicity) of the whole protein and of the loops 2 and 3 (predicted using the GRAVY calculator: http://www.gravy-calculator.de/) / 12-13-14: The propensity to form amyloid structures (predicted using the ZAGG calculator: Tartaglia & Vendruscolo, 2008), the size and the number of amyloid-prone sections and the overall hydrophobicity (predicted using WALTZ: Maurer-Stroh et al., 2010).

*Links between regulation of hydrophobin genes and their properties*

We wanted to know if the hydrophobins that were significantly regulated in a specific condition were characterized by a specific property. For this purpose, we considered two sets of conditions by calculating the following ratios: expression in mycorrhizal tissue/free-living mycelium; and expression in organic matter/mineral medium. We sorted each gene in three categories: significantly upregulated (FDR>0.5 and expression ratio >2); significantly repressed (FDR>0.5 and expression ratio <0.5) and not significantly regulated (FDR<0.5 and expression ratio between 0.5 and 2), and computed the average of each property for these three categories.

*Statistics*

We used an analysis of variance (for each of the 14 predicted structural properties) to check if the proteins that were overexpressed, repressed or not significantly regulated differed significantly in a given property. Normality of the properties data was assessed using the Shapiro-Wilk's test. Properties were transformed when necessary to fit a normal distribution ($\log(x)$, $\sqrt{x}$, $1/x$ or $\arcsin(x)$). When data could not be normalized, analysis of variance was performed using a non-parametric Kruskall & Wallis test. The significance value was corrected for multiple comparisons using the method of Holm-Bonferroni. A Duncan post-hoc test was performed to assess significant differences between regulation groups when the Holm-Bonferroni test was significant. Statistics were performed with R (Ihaka & Gentelman, 1996).

**Results**

*Neighbour-joining tree*

In total, 82 genes potentially coding for hydrophobins were found in the eight mycorrhizal genomes (Table 2). *P. involutus* possessed the highest number of hydrophobins (27). The ERM and ORM fungi had a smaller hydrophobin repertoire (0 to 4 genes) than the ECM ones (5 to 27 genes). Species of a specific exploration type were neither associated with larger nor smaller hydrophobin gene families; there was no trend either in size of the hydprohobin repertoire and host specificity, hydrophobicity of the mycorrhizal organ, or genome size (Table 2). Additionally, we could not identify any significant trends in the relationship between the size of the hydrophobin gene family and the genome size, the land type, exploration type, host specificity and symbiotic organ hydrophobicity as described by Agerer (2001) (Table 2). We aligned the 82 sequences with 20 other hydrophobins from saprotrophic fungi and model hydrophobins.

*Table 2. Ecological characteristics and number of hydrophobin genes found in the investigated genomes. The exploration types and hydrophobicity refer to ectomycorrhizal root tips according to Agerer (2001) and so are not available for ericoid mycorrhizal and orchid mycorrhizal fungus species.*

| Species | Order | Symbiosis | Land type | Exploration type | Host specificity | Hydrophobicity of the mycorrhizal organ | Number of hydrophobin genes in the genome | Genome size (number of genes) | Host for the ECM sample | Age of the ECM sample at harvest (days) | Age of the FLM sample at harvest (days) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Paxillus involutus (B)* | Boletales | Ectomycorrhizal | Forest | Long | Broad | Hydrophobic | 27 | 17968 | *Betula pendula* | 56 | 56 |
| *Hebeloma cylindrosporum (B)* | Agaricales | Ectomycorrhizal | Forest | Short | Broad | Hydrophobic | 19 | 15382 | *Pinus pinaster* | 21 | 6 |
| *Laccaria bicolor (B)* | Agaricales | Ectomycorrhizal | Forest | Fringe | Average | Hydrophilic | 13 | 23120 | *Populus trichocarpa* | 30 | 21 |
| *Suillus luteus (B)* | Boletales | Ectomycorrhizal | Forest | Long | Low | Hydrophobic | 11 | 18316 | *Pinus sylvestris* | 40 | 7 |
| *Piloderma croceum (B)* | Atheliales | Ectomycorrhizal | Forest | Fringe | Average | Hydrophilic | 5 | 21683 | *Quercus robur* | 56 | 56 |
| *Oidiodendron maius (A)* | Onygenales | Ericoid | Peatland, heathland, forest | NA | Low | NA | 4 | 16703 | *Vaccinium myrtillus* | 45 | 45 |
| *Sebacina vermifera (B)* | Sebacinales | Orchid/Ericoid | NA | NA | Low | NA | 3 | 15312 | *Arabidopsis thaliana* | 14 | 14 |
| *Tulasnella calospora (B)* | Cantharellales | Orchid | NA | NA | Low | NA | 0 | 19659 | *Serapias vomeracea* | 30 | 14 |

The neighbour-joining tree showed no clear segregation between fungal species of different ecologies (Figure 1). The 4 hydrophobins of *O. maius* (the only Ascomycete in the tree) were most closely related to the outgroup (HFBII, a model Type-II hydrophobin from the Ascomycete *Trichoderma reesei*). Overall, the tree could be separated into 8 clusters containing more than one hydrophobin; four of these clusters were order-specific (Figure 1). In total, 56 of the 101 genes were found in these order-specific clusters. One cluster (cluster III) was the most diverse, with 3 orders and 9 species represented. The 3 hydrophobins of the Atheliales species *S. vermifera* were clustered together in Cluster V.

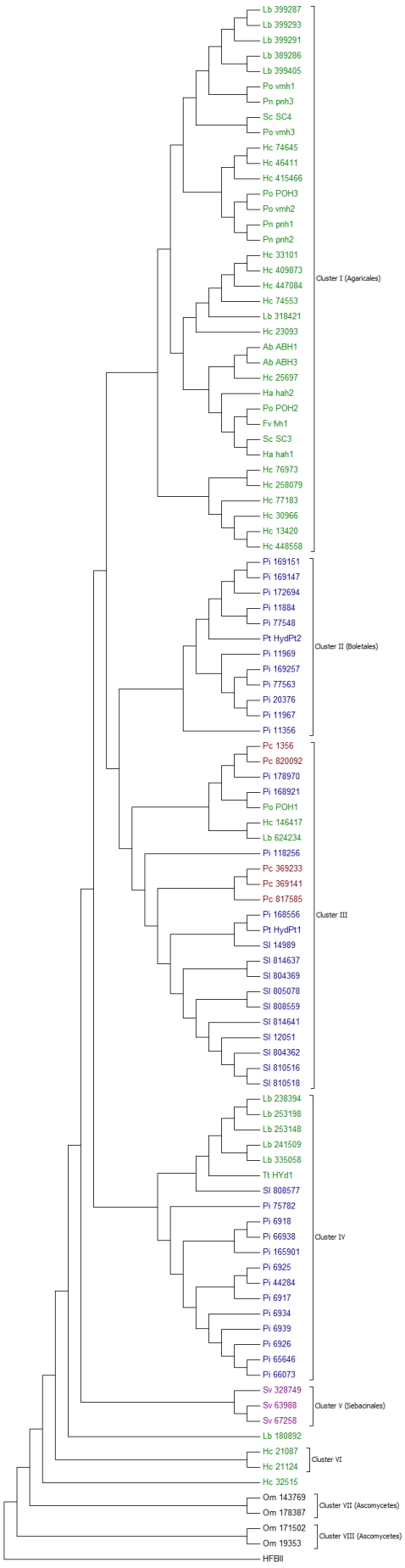


*Figure 1. Phylogenetic reconstruction of the 82 hydrophobin protein sequences from the genomes of L. bicolor (Lb), H. cylindrosporum (Hc), P. involutus (Pi), S. luteus (Sl), P. croceum (Pc), O. maius (Om) and S. vermifera (Sv). Twenty extra hydrophobin protein sequences were added to the analysis, as a type II outgroup (HFBII), or as previously investigated genes or proteins from saprophytic (Po: Pleurotus ostreatus; Pn: Pholiota nameko; Fv: Flammulina velutipes; Sc: Schizophyllum commune; Ab: Agaricus bisporus; Ha: Heterobasidion annosum) or mycorrhizal fungi (Tt: Thelephora terrestris, Pt: Pisolithus tinctorius). The hydrophobin PFAM domain (PF01185) of each sequence has been aligned by CLUSTALW (Gap opening: 0.5, Gap extension: 0.2). Alignment was used to build a neighbor-joining tree with MEGA7. Genes are coloured according to the fungal order to which they belong (green for Agaricales, blue for Boletales, Brown for Atheliales, Purple for Sebacinales, Grey for Russulales, black for Ascomycete orders). Genes also have been grouped by clusters, which were coloured depending on the fungal order that was found dominant within (same color codes as for the genes)..*

In order to identify trends in the genome organization of the hydrophobin family, we performed full-size alignments of the genes for each species, with species-specific neighbour-joining trees using HFBII as an outgroup (Type II hydrophobin) and SC3 as a Type I reference. In 15 cases out of 21, sequences from the same genomic region were also in the same cluster (Figure S1). Alhough we could not accurately compare hydrophobin gene clustering between species because all genomes did not have the same degree of fragmentation, there were monophyletic groups of closely related genes in *P. involutus* (2 clusters of 11 and 5 genes), *H. cylindrosporum* (2 clusters of 4 and 3 genes), *L. bicolor* (2 clusters of 4 and 3 genes) and *P. croceum* (1 cluster of 3 genes). However, in some occurrences, phylogenetically very close genes were in different scaffolds, such as Lb_399293, Pi_17694 and the group Hc_33101-Hc_419873. The last two cases are most likely a result of assembly fragmentation, because they are in a very small scaffold.

To detect possible gene duplication, we performed a topological reconciliation analysis between the overall neighbour-joining tree

of hydrophobins and the phylogenetic tree of the investigated species. In total, 83 duplications and 118 losses were found (Figure S2). In almost all cases the hydrophobin genes that were phylogenetically close within a given species came from duplication events.

*Individual expression levels*

For each species, we compared the expression level of hydrophobin transcripts in growth conditions that should trigger specific biological functions. The hydrophobin expression profile was consistently dominated by only one or two transcripts in each condition, even within large families (Figure 2). These hydrophobin genes were also always among the most expressed of all genes (top 6% in the genes sorted by decreasing expression level in whole transcriptomes of *P. croceum*, top 2% for *S. luteus*, top 0.02% for *P. involutus* and top 0.11% for *H. cylindrosporum*: Kohler et al., 2015, GEO accession GSE63947). For *H. cylindrosporum*, *L. bicolor* and *S. luteus*, the dominant hydrophobins were different between the free-living mycelium and the mycorrhizal tissue conditions. Sometimes, but not always, hydrophobins with low absolute expression levels were highly upregulated in the mycorrhizal tissue (Om171502: 166 times, Pi66073: 63 times, Pi59323: 59 times, Lb318421: 38 times, Pi6934: 21 times). For *H. cylindrosporum* and *S. luteus*, the hydrophobins that were the most expressed in the free-living mycelium were significantly highly repressed in the mycorrhizal tissue.

The hydrophobin transcript profiles differed less between the two substrate conditions than between free-living and mycorrhizal tissues. The most expressed transcripts were the same in both treatments for the four species investigated, but again some little-expressed hydrophobins in the mineral medium were significantly overexpressed in the OM medium (6 out of 27 for *P. involutus*, 4 out of 13 for *L. bicolor*, 5 out of 11 for *S. luteus*, 2 out of 5 for *P. croceum*).

The hydrophobin transcript profile of *S. luteus* was very different between aerial and submerged mycelium; moreover these two profiles differed strongly when compared to the other conditions. The hydrophobins that were most expressed in the submerged growth condition were the least expressed in the free-living mycelium growing in a petri dish. Interestingly, the hydrophobins that were the most overexpressed in the aerial growth condition were also the ones that were the most repressed in the mycorrhizal tissue; these genes also were the most overexpressed in the free-living mycelium condition.

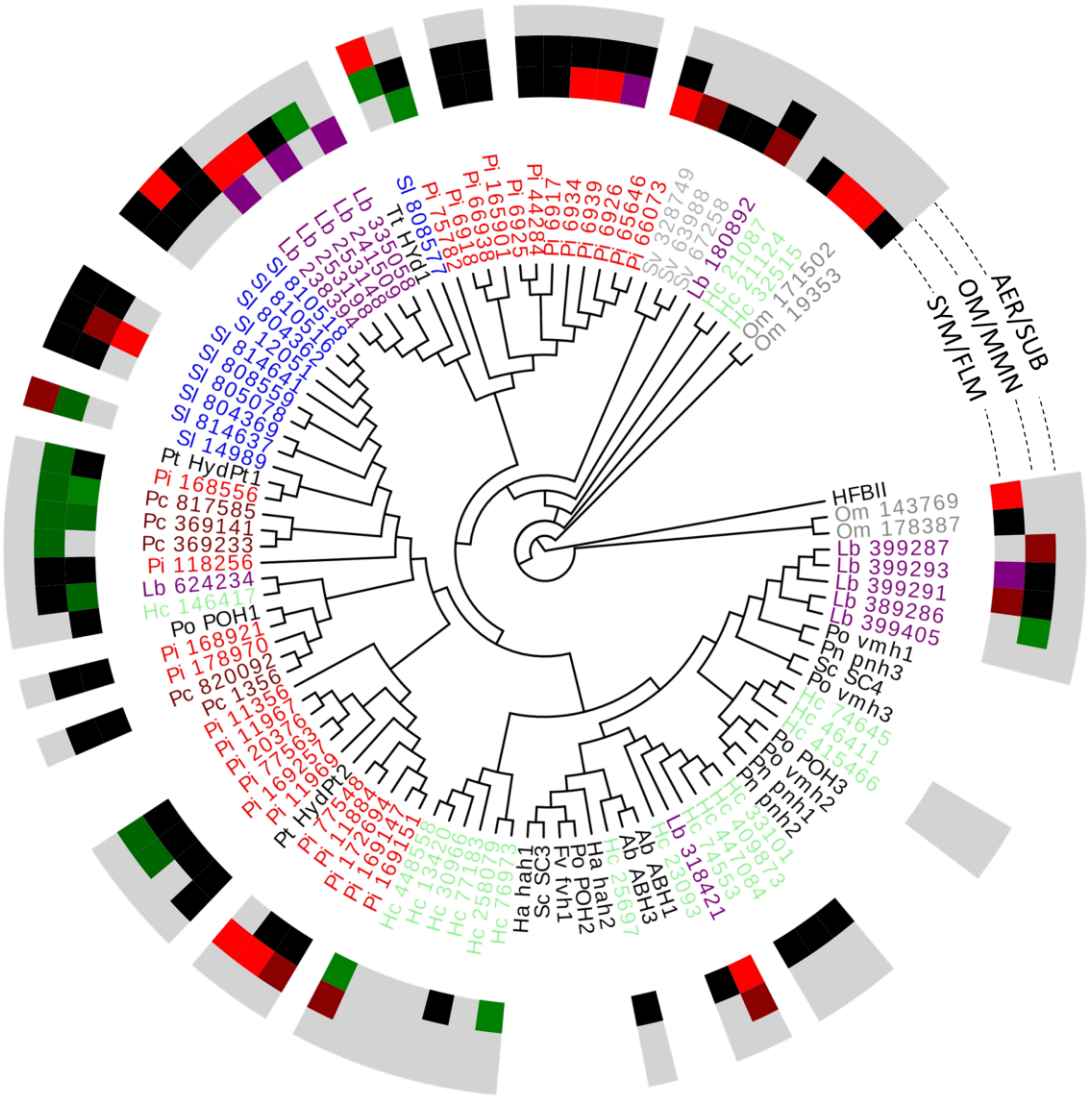


*Figure 2. Regulation of the hydrophobin transcripts in different pairs of conditions (pair 1: FLM: Free-Living Mycelium and SYM: Symbiotic tissue; pair 2: MMN: Mineral medium and OM: Organic Matter medium; pair 3: SUB: Submerged growth and AER: Aerial growth). The hydrophobins are ordered through the same phylogenetic tree displayed in Figure 1. Tree is displayed on Evolview (He et al., 2016). The regulation of each transcript is calculated as follows: SYM/FLM, OM/MMN and AER/SUB; and plotted as a heatmap, with bright red colour indicating an expression ratio above 5, dark red above 2, dark green below 0.5, bright green below 0.2. Genes that were detected only in one condition are in purple and genes for which no significant regulation was found in black (FDR>0.05).*

*Structural properties*

We checked if the proteins that were overexpressed, repressed or not significantly regulated in a given condition differed significantly for their calculated properties. Results showed that all investigated hydrophobins differed over a broad range (coefficient of variation from 17% to 260% depending on the property) for each property (Table 3; see also Table S2 for individual data per hydrophobin). Moreover, hydrophobins that were regulated in

different conditions also had properties that differed significantly among the regulation groups (Table 3). The hydrophobins that were upregulated in the mycorrhizal tissue as compared with free-living mycelium were characterized by a significantly higher exposure of their hydrophilic residues (Figure 3). We've chosen to use the standard cut-off of 2-fold (in addition to a FDR>0.5) to separate significantly regulated and non-regulated genes, but further analysis showed that a 5-fold cut-off would not change the outcome of the comparison.

*Table 3. Predicted functional properties of the hydrophobins and results of the analysis of variance. Wettability N-term: number of O-Glycosylation sites at the N-terminal part of the protein (before the first cysteine). B-sheet: predicted number of beta-sheet structures, A-helix: predicted number of alpha-helix structures, GRAVY: hydrophobicity of the whole protein, GRAVYL2: hydrophobicity of the cys3-cys4 segment, GRAVY L3: hydrophobicity of the cys6-cys7 segment, ZAGG: propensity of the protein to aggregate, WALTZNb: number of the amyloid sections, WALTZ Size: size of the amyloid sections, Hydrophobicity: hydrophobicity at pH4, Charge at pH4: charge of the protein at pH4, . The results of the analysis of variance (ANOVA for normally-distributed variables, Kruskall-Wallis for the others) on each property is given below the table; significant results for Condition 1 (regulation in mycorrhizal tissue as compared with free-living mycelium) and Condition 2 (regulation in organic matter as compared with mineral medium) are highlighted in light grey.*

| | Wettability N-term | Nature of exposed amino acids | | | | | Number of β-sheets and α-helices | | Hydrophobicity | | | Propension to aggregate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | O-Glycosylation sites | exposure of acidic residues | exposure of basic residues | exposure of hydrophobic residues | exposure of hydrophilic residues | exposure of aromatic residues | B-sheet | A-helix | GRAVY | GRAVY L2 | GRAVY L3 | ZAGG | WALTZ Nb | WALTZ Size |
| Average | 15% | 284 | 307 | 1462 | 1754 | 233 | 415 | 432 | 0.493 | 0.564 | -0.225 | -3.78 | 0.513 | 11 |
| Standard error | 0.8% | 25 | 24 | 42 | 74 | 15 | 8 | 8 | 0.025 | 0.043 | 0.062 | 0.18 | 0.085 | 1 |
| Maximum | 42% | 1012 | 1017 | 4166 | 6192 | 1038 | 890 | 917 | 0.957 | 1.432 | 1.483 | -1.13 | 3.000 | 40 |
| Minimum | 3% | 0 | 31 | 725 | 746.978 | 37 | 313 | 335 | -0.049 | -0.608 | -1.283 | -7.34 | 0.000 | 0 |
| Regulation in **mycorrhizal tissue** as compared with **free-living mycelium** (p-value after Holm-Bonferroni correction) | 0.34 | 0.31 | 0.12 | 0.98 | 0.028* | 0.12 | 0.2 | 0.18 | 0.62 | 0.37 | 0.22 | 0.83 | 0.72 | 0.89 |
| Regulation in **organic matter** as compared with **mineral medium** (p value after Holm-Bonferroni correction) | 0.08 | 0.114 | 0.251 | 0.376 | 0.9277 | 0.725 | 0.685 | 0.697 | 0.82 | 0.13 | 0.19 | 0.778 | 0.516 | 0.722 |

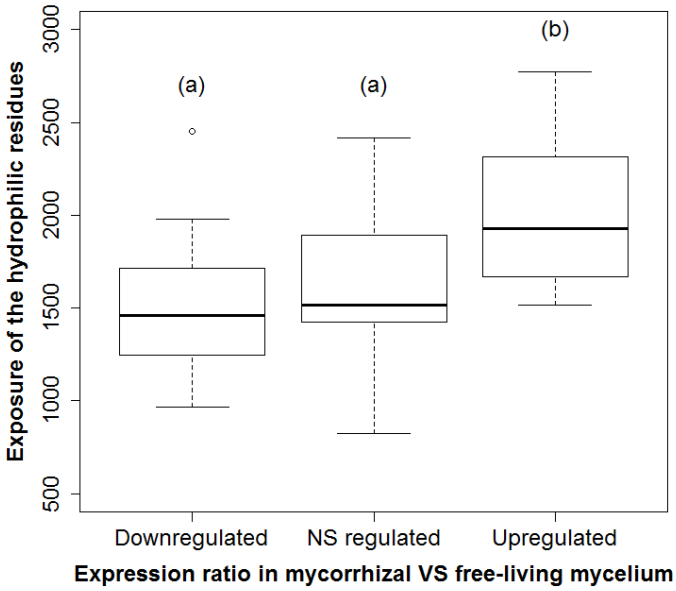


*Figure 3. Boxplot of the difference in the exposure of the hydrophilic residues between downregulated, non-regulated and upregulated hydrophobins in mycorrhizal tissue (as compared with free-living mycelium). The bottom and top of the box are the lower and upper quartiles, respectively; the bar is the median; and the whiskers extend to the most extreme data point which is no more than 1.5 times the length of the box away from the box. The open circle represents a datapoint points that fall outside this range. Each gene was sorted among three categories: significantly upregulated (FDR>0.5 and expression ratio >2); significantly repressed (FDR>0.5 and expression ratio <0.5) and not significantly (NS) regulated (FDR<0.5 and expression ratio between 0.5 and 2). We then used analysis of variance (ANOVA when data had a normal distribution, Kruskall-Wallis analysis otherwise) for the 14 calculated properties to check if the proteins that were overexpressed, repressed or not regulated differed significantly in a given property. The property that was significantly different among expression categories (p<0.05) after Holm-Bonferroni p-value correction for multiple testing is displayed in this figure. Significant differences between expression categories were assessed using a Duncan post-hoc test. Significantly different categories are indicated with different letters.*

**Discussion**

In this work, we investigated the size, sequence, expression levels and calculated structural properties of hydrophobin genes in mycorrhizal genomes. The pattern of expression of hydrophobins generally followed a reciprocal function, with one gene being much more expressed than the others from the same repertoire. The fine tuning of hydrophobin expression revealed in response to our experimental treatments nevertheless was difficult to explain. It appears, however, that variants of the proteins having particular structural features are evoked under certain circumstances. Still, care must be taken in the comparison of the three sets of conditions, because not all species were tested under all, and because of different techniques used (the first two sets used Illumina RNA-seq and the last one qPCR).

*Variation of the hydrophobin repertoire among fungal species*

We found hydrophobin genes in the genomes of five ECM fungi (*L. bicolor, H. cylindrosporum, S. luteus, P. involutus, P. croceum*), two ORM fungi (*T. calospora, S. vermifera*), and one ERM fungus (*O. maius).* All the 82 hydrophobins discoverd were of Type I. This is in accordance with basidiomycetes producing only Type I hydrophobins, whereas Ascomycetes can produce both Type I and Type II (Linder et al., 2005). The only Ascomycete genome investigated (*O. maius*) had only Type I hydrophobins (4 genes). The four *O. maius* hydrophobin genes clustered apart from all Basidiomycete ones, which is consistent with previous reports showing that Ascomycete Type 1 hydrophobins differ from Basidiomycete ones (Whiteford & Spanu, 2002). The size of the hydrophobin gene repertoire was very different between mycorrhizal lifestyles: ORM fungi had 0 (*T. calospora*) and 3 (*S. vermifera*) hydrophobin genes, ERM fungi had 4 (*O. maius*), whereas ECM genomes harboured 5 (*P. croceum*) to 27 (*P. involutus*). The explanation for this large family size in ECM fungi may lie in the overall fungal evolutionary history. Indeed, on average, Ascomycete genomes harbour fewer hydrophobins than Basidiomycetes, which explains the low number of hydrophobin genes in *O. maius*. There was, still, a high variability of gene size repertoire within the investigated basidiomycetes, as already observed in saprophytic basidiomycetes, where most species had large hydrophobin families (around 20 on average) while a few had none at all (Mgbeahuruike et al., 2013). For both symbiotic (the present study) and saprotrophic basidiomycetes (Mgbeahuruike et al., 2013) the size of the hydrophobin repertoire was not correlated with genome size nor ecological preferences. In fact, large hydrophobin families appear to be a feature of the most recent basidiomycota orders: all species in Pucciniomycotina and Ustilaginomycota had no hydrophobins at all (*Melampsora larici-populina*, *Puccinia graminis*, *Rhodothorula graminis*, *Sporobolomyces roseus*, with the exception of *Ustilago maydis* and its only two hydrophobins); within Agaricomycotina, the basal clade of Tremellomycetes did not have hydrophobins either (*Tremella mesenterica*, *Cryptococcus neoformans*) (Mgbeahuruike et al., 2013). Large hydrophobin families are found only in the Agaricomycete clade, with most investigated species having around twenty genes, although a few genomes display a very small hydrophobin repertoire (three in *S. vermifera*, Sebacinales; five in *P. croceum*, Atheliales; three in *Postia placenta*, Boletales), or no hydrophobin at all (*T. calospora*, Cantharellales; and *Stereum hirsutum*, Russulales) (Mgbeahuruike et al., 2013). Therefore, species with well differentiated sporocarps had a larger hydrophobin repertoire than the ones with primitive fruting bodies (Pucciniomycota, Tremellomycota). This can be explained by fruiting bodies requiring partly hydrophobic structures, such as air-filled spaces, gas channels, to prevent waterlogging (Dyer, 2002; Lugones et al., 1999); moreover, different hydrophobins seem to be expressed in different hydrophobic tissues of the fruit body, at least in lichens (Trembley et al., 2007). These proteins have been found to play other roles in a wide variety of fungal species, however, and the formation of elaborate fruiting bodies can only account for a fraction of the total hydrophobin family in many species. Moreover, some other surface proteins such as repellents, have evolved to provide the same functions as hydrophobins, at least in certain conditions (see Kershaw & Talbot, 1998 for a review). That has been observed in *U. maydis*, for example, where surface proteins that were not hydrophobins affected aerial hyphae formation and surface hydrophobicity of mycelium (Wosten et al., 1996); this could explain the absence of hydrophobins from the *T. calospora* genome. Our results therefore confirm that i) the expansion of the hydrophobin gene family occurred mostly within the terminal clades of Basidiomycota, and especially within the class Agaricomycetes; and that ii) presently, the size of the hydrophobin family cannot be associated unambiguously with any specific ecological trait apart from the

formation of complex fruiting bodies. The size of the hydrophobin repertoire was not correlated with fungal host specificity, as results for *T. terreum*, expressing one hydrophobin with a compatible host, could have suggested (Mankel *et al.*, 2002). Interestingly, the 3 hydrophobins of *S. vermifera* were clustered together at the basis of the tree. Hydrophobins do not seem necessary for the establishment of orchid mycorrhizal symbiosis because they were not found in the *T. calospora* genome. *S. vermifera* has a different ecology than *T. calospora*, however, as *S. vermifera* also is reported as an ericoid mycorrhizal fungus. The hydrophobins of this fungus may contribute to its success in ericoid mycorrhiza formation.

Reconciliation analysis of hydrophobin and species trees in the investigated mycorrhizal genomes suggested that many duplication events could contribute to the expansion of this gene family. Plett et al. (2012) already showed that many hydrophobins in two genomes of the mycorrhizal fungus *L. bicolor* were consequences of duplication events followed by positive selection. Our results confirmed that in 42% of the cases, hydrophobins evolved through gene duplication events. Despite the substantial variation in hydrophobin sequences, the genes that were physically clustered in the same region of the genome most often coded for proteins of high similarity. This was especially clear in the 1705-2019K region of the scaffold 1 in the *P. involutus* genome, which contained 11 very similar hydrophobin sequences. This trend was found even in the genome of *O. maius*, which had only 4 hydrophobin genes, but two in proximity in the same scaffold. This is especially interesting considering that in fungal genomes rearrangments are frequent, resulting in high gene divergence even within genera. Hence, the conservation of genomic localization could be due to a high frequency of duplication of hydrophobin genes. The relatively low number of species investigated in this work, however, does not allow us to test this hypothesis.

*Hydrophobin expression levels*

Our interpretation of results is hampered partially by not all conditions having been tested for all species. This made cross-comparisons between pairs of conditions impossible. There was, however, one general trend: hydrophobin expression within a species repertoire always followed a reciprocal function: a few highly expressed genes vs. many, little-expressed ones. The mRNAs of the highly expressed hydrophobin genes also were among the most abundant mRNAs of the whole transcriptome. Even though we cannot conclude the exact role of these dominant hydrophobins based on our dataset, such high expression levels suggest that these peptides are important structural proteins (for example, helping cell wall maturation: see Whiteford & Spanu, 2002). The sequence of these “structural” hydrophobins, however, were very different among species.

We also observed differences in hydrophobin gene regulation between treatments. The pattern of hydrophobin expression was indeed much more similar between organic and mineral medium than between symbiotic and free-living mycelium. This can be explained by the substantial morphological and physiological differences between an ectomycorrhizal root tip and mycelium growing in a petri dish, as compared with the relatively minor ones between mycelium growing atop two liquid media (regardless of its composition). The same remark stands for the large differences we observed in hydrophobin expression between aerial and submerged mycelium of *S. luteus*. Consequently, the most expressed hydrophobins in the mineral medium remained the most expressed ones in the organic condition for the four species investigated.

The fine-tuning of the expression of hydrophobin genes in each condition appeared complex, because it differed a lot between species, without following a pattern that would be explained by species traits or ecology. For all but three species (*S. luteus, L. bicolor* and *H. cylindrosporum*) the most transcribed hydrophobin was the same in

the symbiotic and in the free-living tissues. But these three species did not share any unique feature (they all have an hydrophobic mantle and rhizomorphs, but so does *P. involutus*; they differ widely in their host specificity, genome size, and were associated with both deciduous and coniferous hosts). The most expressed hydrophobins always had very dissimilar amino acid sequences, whatever the treatment. Some hydrophobins that have low expression levels, however, were significantly regulated either in symbiotic tissue or in organic matter medium; but again there was no clear ecological pattern that could explain these results.

In order to confirm these results and assess the actual hydrophobin functions, further cross-validation experiments would be needed, such as comparing submerged mycelium with mycorrhizal tissues in hydroponic culture, or testing different dilutions of organic nutrients, and running surfactant activity assays of extracted hydrophobin mixtures to validate their role as surfactants.

*Structural properties*

Although the hydrophobins that were overexpressed in a given treatment differed a lot in their sequences, they had structural properties in common in two different sets of conditions. Hydrophobins that were overexpressed in mycorrhizal tissues as compared with free-living mycelium showed a significantly higher number of exposed hydrophilic residues. Because the hydrophilic side of the protein is the one anchored on polysaccharides from the fungal cell wall (Whiteford & Spanu, 2002), this suggests that the proportions of hydrophilic residues (S, T, N, Q) of this side is larger in hydrophobins overexpressed in the mycorrhizal tissue (our results also show that the exposure of the hydrophobic patch is not significantly affected). Similar observations of high proportions (especially of Q and T) have been made, though on the N-terminal part of the protein only (Whiteford & Spanu, 2002). Hence, we speculate that this could mediate a stronger attachment to the fungal polysaccharides in the mycorrhizal tissue as compared with the free-living mycelium, and result in a smaller proportion of secreted hydrophobins. Considering the complexity of the structural resolution of hydrophobins (Lienemann et al., 2013), however, we cannot interpret these data further without an in-depth proteomic study.

**Conclusion**

Analysis of the gene repertoire and regulation of mycorrhizal hydrophobins show that this family expanded in the most recent orders, to a significant extent thanks to gene duplication. The expression patterns always showed one gene that was more highly expressed than others, which suggests a role as a structural protein. Several genes were highly regulated in the symbiotic tissue (as compared with the free living tissue), in the organic matter medium (as compared with the mineral one), or in the aerial mycelium (as compared with the submerged one). They were coding for proteins of very different sequences but which were characterized by significantly different calculated structural properties.

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