



Phylogenetic, genomic organization and expression analysis of hydrophobin genes in the ectomycorrhizal basidiomycete *Laccaria bicolor*

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ABSTRACT

Hydrophobins are morphogenetic, small secreted hydrophobic fungal proteins produced in response to changing development and environmental conditions. These proteins are important in the interaction between certain fungi and their hosts. In mutualistic ectomycorrhizal fungi several hydrophobins form a subclass of mycorrhizal-induced small secreted proteins that are likely to be critical in the formation of the symbiotic interface with host root cells. In this study, two genomes of the ectomycorrhizal basidiomycete *Laccaria bicolor* strains S238N-H82 (from North America) and 81306 (from Europe) were surveyed to construct a comprehensive genome-wide inventory of hydrophobins and to explore their characteristics and roles during host colonization. The S238N-H82 *L. bicolor* hydrophobin gene family is composed of 12 genes while the 81306 strain encodes nine hydrophobins, all corresponding to class I hydrophobins. The three extra hydrophobin genes encoded by the S238N-H82 genome likely arose via gene duplication and are bordered by transposon rich regions. Expression profiles of the hydrophobin genes of *L. bicolor* varied greatly depending on life stage (e.g. free living mycelium vs. root colonization) and on the host root environment. We conclude from this study that the complex diversity and range of expression profiles of the *Laccaria* hydrophobin multi-gene family have likely been a selective advantage for this mutualist in colonizing a wide range of host plants.

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1. Introduction

The colonization of living host tissue by mutualistic organisms is a delicately balanced process. One mutualistic relationship of global importance is the interaction between forest trees and soil-borne ectomycorrhizal (ECM) fungi where the fungus provides a wide range of nutrients to the plant in return for photosynthetically fixed carbon (Martin, 2007). Colonization of plant tissues likely requires a multi-pronged approach on the part of the fungus as it must form the symbiotic interface between the two partners while protecting the invading hyphae from host defenses. A group of proteins, named MYCORRHIZA-INDUCED SMALL SECRETED

PROTEINS (MiSSPs), have been proposed to aid in these two processes (Martin et al., 2008; Plett and Martin, 2011). MiSSPs are a disparate group of proteins that share some common traits; they are small (<300 amino acids), they are often cysteine-rich and they are induced by the symbiotic process. Recently we characterized MiSSP7, a gene highly induced in *Laccaria bicolor* by the colonization process (Plett et al., 2011). We were able to demonstrate that production of the MiSSP7 protein was necessary for *L. bicolor* to colonize the host plant *Populus trichocarpa*. Most other MiSSPs have no known biochemical function. One group of MiSSPs, however, that have known functions in pathogenic and saprotrophic fungi, and that are thought to have important roles during the symbiotic colonization of plant tissues as well, are the hydrophobins. Hydrophobins are small secreted, moderately hydrophobic, self-assembling polypeptides with a conserved distribution of eight cysteine residues that are crucial for proper protein folding (Sunde et al., 2008). They are involved in many aspects of fungal biology where adhesion occurs (Wessels et al., 1991; Kershaw and Talbot, 1998; Duplessis et al., 2001; Wösten, 2001; Walser et al., 2003; Linder et al., 2005). Hydrophobins have been proposed to have a protective role in resistance to desiccation and plant

Abbreviations: MiSSP(s), MYCORRHIZA-INDUCED SMALL SECRETED PROTEIN(S); TE(s), transposable element(s); ECM, ectomycorrhizal; LG, linkage group; sc, scaffold; FLM, free-living mycelium.

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excreted defensive compounds (Temple et al., 1997; Wessels, 1997; Kershaw and Talbot, 1998; Wösten et al., 1996). Production of hydrophobins is up-regulated in several pathogenic interactions during plant colonization (Talbot et al., 1993; Zhang et al., 1994; Kazmierczak et al., 1996; Talbot et al., 1996; Holder and Keyhani, 2005). Hydrophobins are also thought to be important in mutualistic mycorrhizal and lichen symbioses based on expression profiles during mutualistic interactions (Duplessis et al., 2001, 2005; Tagu et al., 1996, 1998, 2001; Voiblet et al., 2001; Trembley et al., 2002a,b; Scherrer et al., 2002; Scherrer and Honegger, 2003; Le Quééré et al., 2006; Rajashekar et al., 2007). In these symbiotic structures, hydrophobins remain in the apoplast and localize to the cell wall surface of the outer mantle and the hyphae of the Hartig net (Tagu et al., 2001; Mankel et al., 2002). The full range of functions of hydrophobins in the establishment of ectomycorrhizal root tips still remains unclear, although many roles have been proposed including binding of hyphae to the host root, aggregation of hyphae to form the mantle and aiding in root penetration (Tagu et al., 1998). The small size of these proteins, their induction by the mycorrhization process, their secretion and their proposed role in mediating host:fungal interactions would suggest that these proteins form a subclass of MiSSPs whose role is principally in the root apoplastic space.

As very little is known about apoplastic MiSSPs, the aim of this study was to establish a comprehensive, genome-wide inventory of hydrophobin genes from two different genomes of the ECM fungus *L. bicolor* (strain S238N-H82 and strain 81306). These two strains were chosen as they originate from two different continents (North America and Europe) and would last have shared an ancestor approximately 5.5–6.5 million years ago (M. Ryberg; personal communication). We identify, here, those genes induced by the mycorrhization process. We identified 12 hydrophobin genes in the reference S238N-H82 strain and nine hydrophobin genes in the 81306 strain whose products all conformed to known hydrophobin class I sequences. Genome organization, phylogeny and evolution of these hydrophobin genes are discussed.

2. Materials and methods

2.1. Fungal cultures, ectomycorrhizal synthesis and RNA extraction

For genomic extraction, free-living mycelium (FLM) of *L. bicolor* (Maire) P.D. Orton S238N-H82 (a sib-monokaryon of S238N; Oregon, USA) and 81306 (dikaryon; Barbaroux, France) were grown in liquid high-glucose Pachlewski medium (Di Battista et al., 1996) for 3 weeks at 24 °C before harvesting. Genomic extraction was performed as described by Martin and colleagues (2008). FLM of *L. bicolor* S238N (dikaryon; Oregon, USA) used as a control in expression analyzes were grown on cellophane membranes on high-glucose Pachlewski medium for 3 weeks after which the proliferating hyphal tips at the colony edge were isolated, frozen in liquid nitrogen and used for RNA extraction. The sequenced monokaryon S238N-H82 was not used for mycorrhization tests as monokaryons of *Laccaria* do not colonize the roots of *Populus* sp. Ectomycorrhizal root tips were harvested from contacts between *L. bicolor* S238N (dikaryon) and either Douglas fir (*Pseudotsuga menziesii* var. *menziesii*), *P. trichocarpa* Torr. & A. Gray, *Populus deltoides* W. Bartram ex Marshall or *P. trichocarpa* × *P. deltoides* clone 545 (code 54) grown under conditions as described by Frey-Klett and colleagues (1997) for Douglas fir or as per Plett and colleagues (2011) for all poplar species. Fruiting bodies of *L. bicolor* S238N were collected beneath 9 month old Douglas fir seedlings or 6 months old poplar saplings inoculated with *L. bicolor* S238N grown under glass house conditions. Whole, stage 6 fruiting bodies of similar size (*L. bicolor* fruiting bodies develop in six stages; Lucic

et al., 2008) were chosen and used in the analysis presented in this paper. One fruiting body was used per biological replicate.

2.2. In silico genome automatic annotation and manual curation of hydrophobin genes

Gene prediction for the *L. bicolor* S238N-H82 monokaryon genome (v. 2.0) was based on Sanger improved genome assembly, expressed sequence tags (EST) sequenced with Sanger, 454 sequencing, and Illumina RNA-Seq, coding sequence completeness and homology to a curated set of proteins. Genomic sequence and annotated genes are available at the JGI *L. bicolor* portal (<http://genome.jgi.doe.gov/Lacbi2/Lacbi2.home.html>). The *L. bicolor* 81306 dikaryon genome was sequenced using the Illumina HiSeq 2000 (2 × 100 bp paired end reads), and image analysis and data extraction were performed using Illumina RTA 1.7.48. Raw sequence data have been deposited in the Sequence Read Archive (SRA) database with accession number NCBI SRA049765.1. Reads were assembled using Velvet (Zerbino and Birney, 2008). Simulated long pairs were created from the Velvet assembly using wgsim and fed into ALLPATHS-LG along with the original reads to produce the draft assembly. The *L. bicolor* 81306 genome assembly of 5422 contigs contained 95.3% of the 19,036 *L. bicolor* S238N-H82 gene models. The 890 missing genes are coding for transposon-protein fragments or hypothetical proteins. A tBLASTn search (cutoff e-value of $\leq 1e-5$) using the protein sequences of the Core Eukaryotic Genes (CEG) (Parra et al., 2009) showed that 243 (98%) of the 248 CEG proteins were found in the current *L. bicolor* 81306 draft assembly, suggesting that most of the coding space of *L. bicolor* 81306 was covered. *L. bicolor* S238N-H82 hydrophobins were identified using four approaches: (1) comparison to known hydrophobin protein sequences from *Coprinopsis cinerea* and other basidiomycetes (*Agaricus bisporus*, *Agrocybe aegerita*, *Dictyonema glabratum*, *Pleurotus ostreatus* and *Pisolithus tinctorius*) by querying using BLASTp; (2) these basidiomycete hydrophobins were also used in tBLASTn queries against the *L. bicolor* v2.0 genome; (3) gene models with a predicted hydrophobin domain (IPR001338) were identified using INTERPROSCAN; (4) finally, the assemblies were scanned using an Hidden Markov Model (HMM) to identify the hydrophobin signature (Class I hydrophobins: C-X₅₋₈-C-C-X₁₇₋₃₉-C-X₈₋₂₃-C-X₅₋₆-C-C-X₆₋₁₈-C-X₂₋₁₃; Class II hydrophobins: C-X₉₋₁₀-C-C-X₁₁-C-X₁₆-C-X₈₋₉-C-C-X₁₀-C-X₆₋₇). All detected hydrophobin gene models were inspected manually and edited when required. Hydrophobin-like sequences in *L. bicolor* 81306 were identified using tBLASTn query followed by manual annotation. All hydrophobin genes found by this method were full length. Signal peptides were detected via SignalP 4.0 using default parameters (Nielsen and Krogh, 1998; Bendtsen et al., 2004). Hydrophathy plots were generated using the scale set by Kyte and Doolittle (1982) on the ProtScale ExpASY Proteomics Server (Gasteiger et al., 2005).

2.3. Sequence alignment and phylogenetic analysis

Predicted protein sequences from the present genome surveys were aligned with a broad selection of other basidiomycete hydrophobin sequences using the MUSCLE alignment program using the default settings (Edgar, 2004). The aligned sequences were exported to the MEGA5 program (Tamura et al., 2011) and a Maximum Likelihood phylogenetic tree was generated using protein pair-wise distances with bootstrap resampling of 500 times. All pairwise values of nonsynonymous substitutions per nonsynonymous site (Dn) and synonymous substitutions per synonymous site (Ds) were calculated using MEGA5 (Tamura et al., 2011). The ratio Dn/Ds provides an estimate of the evolutionary rate of amino acid substitutions as well as standard errors by bootstrap resampling (Miyata and Yasunaga, 1980; Felsenstein, 1985).

2.4. Transposon survey

To determine if full length transposable elements (TE's) were within the vicinity of the hydrophobin genes, the 245 full length TE's found within the genome of *L. bicolor* (Martin et al., 2008) were blasted against both the genome of *L. bicolor* S238N-H82 and of *L. bicolor* 81306. It must be noted that Velvet assembly of Illumina data used in the sequencing of the 81306 strain does not include most TE/repeat rich regions of the genome, therefore we could not accurately localize TE's within this draft genome. Only full length TE's within a 120 kb window around the hydrophobin genes of the S238N-H82 genome were considered as these are the youngest TE's and most likely to be active.

2.5. Transcript profiling

A quantitative PCR approach was used to follow the expression of the hydrophobin genes in mycorrhizal root tips and in fruiting bodies in comparison to gene expression in free-living mycelium. The effect of nutrient starvation on the expression of hydrophobins was followed using custom NimbleGen *L. bicolor* oligoarrays (Martin et al., 2008). Due to close sequence similarity *LbH9* and *LbH10* had to be considered as one gene. Because mycorrhization systems using the *L. bicolor* strain S238N are the most developed, we used only this strain for mycorrhization trials. Three biological replicates of between 50 and 100 mg of free-living mycelium *L. bicolor* S238N (used to set the basal expression of each hydrophobin) or 12 week old mycorrhizal root tips from Douglas fir (*P. menziesii*), *P. trichocarpa*, *P. deltooides* or *P. trichocarpa* × *P. deltooides* clone 545 or stage 6 fruiting bodies of *L. bicolor* in association with either Douglas fir or *Populus sp.* were harvested and frozen in liquid nitrogen and used for RNA extraction. For nutrient deprivation experiments, *L. bicolor* colonies (grown on cellophane membranes) were started on MMN media for 15 days after which they were transferred to fresh MMN media (as a control) or to MMN with a 10 times reduction in all major macro-elements; to MMN with a 10 times reduction in the quantity of glucose; or onto agar medium supplemented with the same nutrients used to fertilize our mycorrhization experiments (2.5 mM KNO₃, 0.8 mM KH₂PO₄, 1 mM MgSO₄·7H₂O, 2.3 mM Ca(NO₃)₂·4H₂O, 23 μM H₃BO₃, 4.6 μM MnCl₂·4H₂O, 0.4 ZnSO₄·7H₂O, 0.09 μM (NH₄)₂MoO₄, 0.18 μM CuSO₄·5H₂O, 20 μM FeNaEDTA, pH 5.8). Total RNA was extracted using the RNAeasy kit (Qiagen; Courtaboeuf, France) as per the manufacturer's instructions with the addition of 20 mg polyethylene glycol 8000/mL to the RLC extraction buffer. An on-column DNA digestion step with DNase I (Qiagen) was also included to avoid DNA contamination. RNA quality was verified by Experion HighSens capillary gels (Bio-Rad; Marnes-la-Coquette, France).

Synthesis of cDNA from 1 μg of total RNA was performed using the iScript kit (Bio-Rad) according to manufacturer's instructions. Transcript profiling was performed using custom NimbleGen oligoarrays as described (Martin et al., 2008). A Student *t*-test with false discovery rate (FDR) (Benjamini–Hochberg) multiple testing correction was applied to the data using the ARRAYSTAR software (DNA-STAR). Transcripts with a significant *p*-value (<0.05) and ≥2.5-fold change in transcript level were considered as differentially expressed. The complete expression dataset is available as series (accession number GSE35556). A Chromo4 Light Cycler Real-time PCR was used for real-time PCR analyzes on three biological replicates (with two technical replicates per biological replicate) using the SYBRGreen Supermix following the manufacturer's instructions (Bio-Rad). Fold changes in gene expression between mycorrhizal and free-living mycelium were based on ΔΔCt calculations according to Pfaffl (2001). The data was normalized with two reference genes in each experiment: *ELONGATION FACTOR3* (JGI Protein ID: 293350) and *METALLOPROTEASE* (JGI Protein ID: 245383). These

reference genes were chosen due to stability of expression during the mycorrhization process as determined by microarray analysis. A Student's two tailed independent *T*-test was used to determine the significance of the results (*p* < 0.05).

3. Results

3.1. *L. bicolor* encodes a wide variety of Class I hydrophobin genes

Using a combination of annotation procedures 14 hydrophobin gene models from *L. bicolor* S238N-H82 that met the criteria of a classical hydrophobin (predicted secretion signal, proper cysteine spacing, similarity to known hydrophobins) were identified in the genome assembly v1.0 and named *LbH1* to *LbH14*. Of these gene models, *LbH4* and *LbH6* were merged into one gene (hereafter referred to as *LbH6*) as they showed the same coordinates using the *L. bicolor* version 2.0 genome assembly. *LbH7* is a pseudogene with a number of nonsense point mutations, including frameshifts, and therefore is not considered to be a functional gene and was renamed *LbHx*. Hence, the homokaryotic (haploid) *L. bicolor* S238N-H82 encodes 12 hydrophobins (Fig. 1; Table 1). To determine the conservation of hydrophobins within the *L. bicolor* clade, we also annotated the hydrophobin genes of *L. bicolor* 81306, a strain originating in Europe rather than North America and separated from the reference strain by approximately 5.5–6.5 million years of evolution (M. Ryberg; personal communication). This genome only coded for nine hydrophobin genes (Fig. 1; Table 1). As in *L. bicolor* S238N-H82, *LbH7* was degenerate and did not code for a full length protein. There also appeared to be fewer duplications as *LbH1* and *LbH5* mapped to the same gene in *L. bicolor* 81306 and were thus annotated as one gene (*LbH5*₈₁₃₀₆). Similarly *LbH8*, *LbH9* and *LbH10* were annotated as one gene rather than three (called *LbH8*₈₁₃₀₆).

Of the 12 *L. bicolor* S238N-H82 predicted hydrophobin proteins, 11 possessed the eight conserved cysteines of canonical class I hydrophobins and a predicted signal peptide (confidence levels between 0.886 and 0.983; Table 1). The twelfth hydrophobin-like gene of *L. bicolor* S238N-H82, *LbH14*, had only seven of the eight cysteines normally found in the consensus sequence of class I hydrophobins due to a deletion of the fifth conserved cysteine. This same cysteine was also deleted in *LbH14*₈₁₃₀₆ of the *L. bicolor* 81306 genome. Thus, the consensus sequence of *LbH14* is: X₁₄₆-C-X₆-C-C-X₃₁-C-X₁₁-[]-X₅-C-C-X₁₂-C-X₇. Of the 12 gene models in *L. bicolor* S238N-H82 and the nine genes in the 81306 strain, *LbH3*, *LbH11* and *LbH14* encode additional cysteine residues (Fig. 1). *LbH14* also has a greatly extended N-terminal region containing 7 copies of the seven amino acid repeat P₇₀ X₆₂ I/T₈₅ T₉₂ T₁₀₀ X₅₄ I/T₇₇ (Table 2). *LbH14*₈₁₃₀₆ had the same repeat sequence, but only four repeats were present. Hydropathy patterns of all 12 hydrophobin genes from *L. bicolor* S238N-H82 and all nine hydrophobins of *L. bicolor* 81306 conformed to class I hydrophobins, with a small string of hydrophilic amino acids following each of the two cysteine doublets (Suppl. Fig. 1). In most cases, these hydrophilic domains were very short and these regions were not highly hydrophilic. Meanwhile, in between the cysteine doublets, both strains of *L. bicolor* exhibited very long regions of high hydrophobicity (e.g. *LbH1-6*). Therefore, in general, the hydrophobins of *L. bicolor* all conform to classical class I hydrophobins.

3.2. Gene structure and genomic organization of the predicted hydrophobin proteins

The hydrophobin gene sequences varied from 506 bp for *LbH13* to 1064 bp for *LbH14* (Table 1). Multiple protein sequence alignment and intron analysis of the *LbH* genes revealed the existence of two main subgroups in *L. bicolor* and three genes which did

Table 1
General characteristics of the hydrophobins of *L. bicolor*. Protein IDs are based on JGI annotations.

Hydrophobin	Protein ID	Linkage Group	Scaffold	Length (Bases)	SignalP Value	Consensus sequence
LbH1	399293	LG4		565	0.947	X41-C-X6-C-C-X33-C-X13-C-X5-C-C-X12-C-X7
LbH2	624234	LG1		895	0.969	X60-C-X6-C-C-X31-C-X13-C-X5-C-C-X12-C-X8
LbH2 ₈₁₃₀₆			731	660	0.972	X60-C-X6-C-C-X31-C-X13-C-X5-C-C-X12-C-X8
LbH3	399291	LG2		671	0.975	X37-C-X6-C-C-X28-C-X17-C-X5-C-C-X5-C-X53
LbH3 ₈₁₃₀₆			240	666	0.973	X37-C-X6-C-C-X28-C-X17-C-X5-C-C-X5-C-X43
LbH5	399287	LG2		566	0.948	X37-C-X6-C-C-X32-C-X13-C-X5-C-C-X12-C-X7
LbH5 ₈₁₃₀₆			240	566	0.953	X40-C-X6-C-C-X32-C-X13-C-X5-C-C-X12-C-X7
LbH6	389286	LG2		552	0.983	X40-C-X6-C-C-X32-C-X13-C-X5-C-C-X12-C-X7
LbH6 ₈₁₃₀₆			240	554	0.983	X40-C-X6-C-C-X32-C-X13-C-X5-C-C-X12-C-X7
LbH8	253148			15	0.957	X27-C-X6-C-C-X32-C-X13-C-X5-C-C-X13-C-X24
LbH8 ₈₁₃₀₆				197	0.956	X27-C-X6-C-C-X32-C-X13-C-X5-C-C-X13-C-X19
LbH9	253198			15	0.962	X27-C-X6-C-C-X32-C-X13-C-X5-C-C-X13-C-X7
LbH10	238394			15	0.958	X27-C-X6-C-C-X32-C-X13-C-X5-C-C-X13-C-X7
LbH11	180892			20	0.947	X28-C-X8-C-C-X34-C-X9-C-X5-C-C-X12-C-X7
LbH11 ₈₁₃₀₆			297	480	0.946	X28-C-X8-C-C-X34-C-X9-C-X5-C-C-X12-C-X7
LbH12	241509	LG4		509	0.962	X28-C-X6-C-C-X32-C-X13-C-X5-C-C-X12-C-X5
LbH12 ₈₁₃₀₆	1951		1951	509	0.957	X28-C-X6-C-C-X32-C-X13-C-X5-C-C-X12-C-X5
LbH13	335058	LG4		506	0.972	X28-C-X6-C-C-X32-C-X13-C-X5-C-C-X12-C-X5
LbH13 ₈₁₃₀₆	2111		2111	503	0.973	X28-C-X6-C-C-X32-C-X13-C-X5-C-C-X12-C-X5
LbH14	318421	LG3		1064	0.88	X146-C-X6-C-C-X31-C-X11-[]-X5-C-C-X12-C-X7
LbH14 ₈₁₃₀₆			140	1056	0.941	X146-C-X6-C-C-X31-C-X11-[]-X5-C-C-X12-C-X7

Table 2
Comparison of N-terminal repeats of *L. bicolor* LbH14 and LbH14₈₁₃₀₆ to other basidiomycete hydrophobins with extended N-terminal regions.

Organism	Hydrophobin	Repeat length	Repeat sequence	Copies
<i>Laccaria bicolor</i> S238N-H82	LbH14	7	P70X62I/T85T92T100X54I/T77	7
<i>Laccaria bicolor</i> 81306	LbH14 ₈₁₃₀₆	7	P70X62I/T85T92T100X54I/T77	4
<i>Phanerochaete chrysosporium</i>	PcH5	4	K50T83V83T100	6
<i>Phanerochaete chrysosporium</i>	PcH6	4	P50T100V50T100	4
<i>Phanerochaete chrysosporium</i>	PcH19	4	P50T100V50T75	4
<i>Phanerochaete chrysosporium</i>	PcH7	5	G78I78L89P100T/S89	9
<i>Phanerochaete chrysosporium</i>	PcH17	5	G83I/L92L96P100T/S100	24
<i>Ustilago maydis</i>	Hum3	36	N100A100P100D100F100D100V57V100K86N100S100N86Q100V86L100P100I100Q86A100T100A86A100L86L100S100Q71I/V100A86N100G86Q100S86V86E86K86R100	7
	Hum3	30	S40V90D/E90N100T/S80N90Q90I/V80I/V/L90P100I/V90Q60A40T/S80L80A90A80L70S100Q30I/V/L100V/L70N90S40Q90K80A80T/S40R/K90R/K70	10

As compared to mycorrhizal root tips, far fewer hydrophobins were regulated in fruiting bodies, despite the crucial role for hydrophobins in fruiting body formation (Wessels et al., 1991; Fig. 4D). In fruiting bodies harvested from Douglas fir hosts and poplar, LbH5 and LbH14 were induced while LbH6 and LbH11 were repressed. LbH1 was significantly induced in fruiting bodies from *L. bicolor* Douglas fir associations while LbH11 was only induced in *L. bicolor* poplar fruiting bodies (Student's *T*-test; $p < 0.05$). These differences may be related to the different physiology of alternate plant hosts and the nutrient fluxes derived from these interactions or may be due to minute differences in the developmental stage of each fruiting body assayed. Further, we found in a range of different nutrient starvation experiments (e.g. reduction in macro-elements, reduction in carbon source), that expression of the hydrophobin genes was not significantly affected (data not shown).

4. Discussion

The first ectomycorrhizal (ECM) fungal genomes (Martin et al., 2008, 2010) gave meaningful insight into the evolution of mutualistic fungi from saprotrophic ancestors (Eastwood et al., 2011). Based on phylogenetic and genomic evidence it has been proposed

that the evolution of the mutualistic lifestyle resulted, in part, through the expansion of small secreted effector proteins induced by mycorrhization (MiSSPs) that alter host biology, that make up part of the symbiotic interface and that counter-act plant defenses (Martin et al., 2008; Plett and Martin, 2011). Hydrophobins are a class of small secreted proteins that have a wide range of functions in most fungi – both those that interact with living organisms and those that live on inert media. These include aid in attachment to different substrates, in aggregation of hyphae to one-another and in preventing desiccation during the formation of fruiting bodies, to name but a few (Wessels, 1996, 1997; Wösten, 2001). The catalog of hydrophobins encoded by different fungal genomes varies widely, with no direct correlation between the absolute number of hydrophobins encoded by a certain genome and the lifestyle of the fungus. Hydrophobins have been largely characterized in saprotrophic and pathogenic fungal systems (Wessels et al., 1991). There are two classes of hydrophobins, class I which is encoded by fungi from both the basidiomycetes and ascomycetes and class II which is exclusively found within the ascomycetes. Of the two classes, class I hydrophobins are considered to be more stable (Askolin et al., 2006) and to be crucial for the formation of aerial hyphae and colonization (Askolin et al., 2005, 2006). Class I

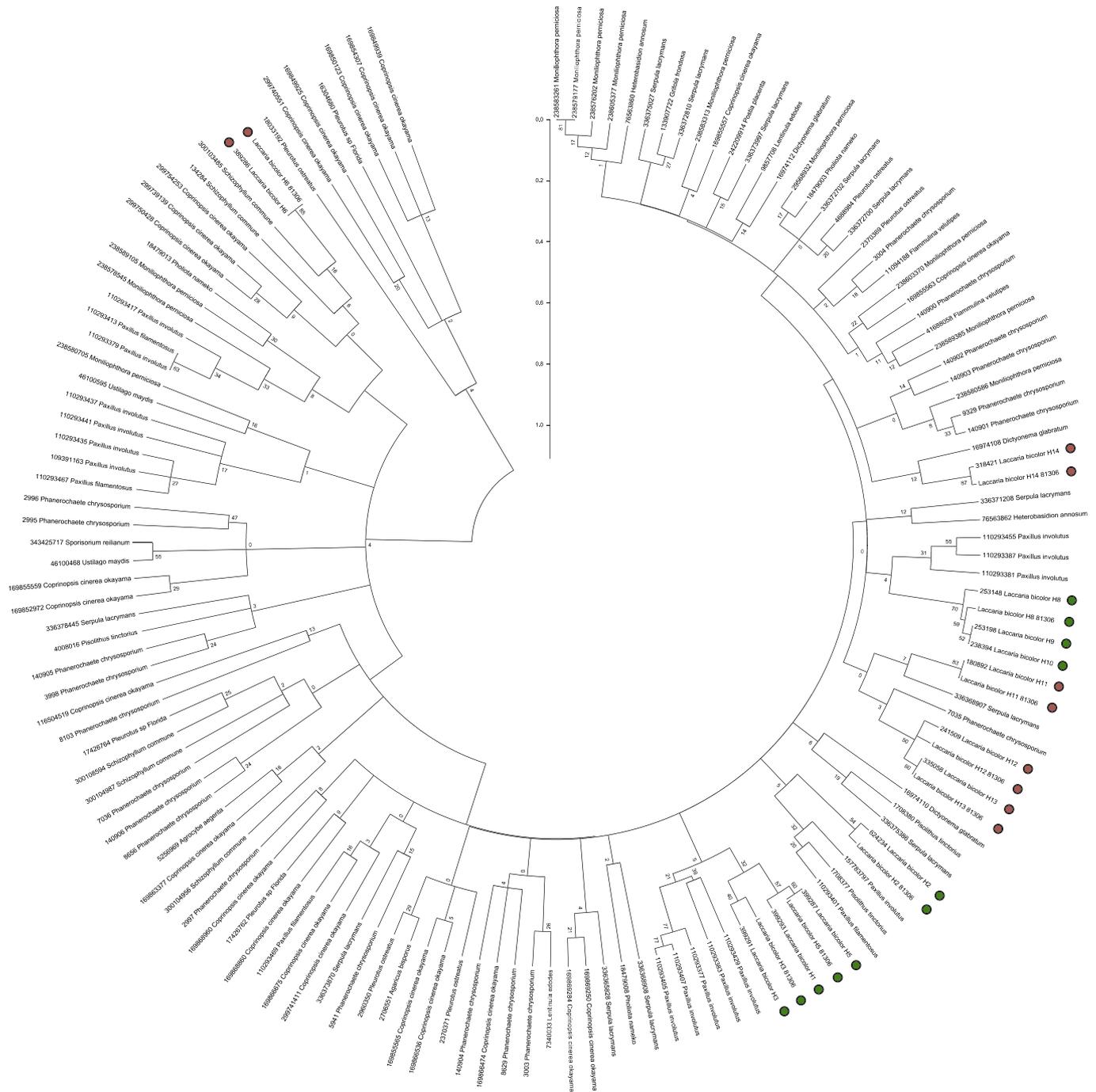


Fig. 2. Phylogenetic relationship of *L. bicolor* hydrophobins to other basidiomycete hydrophobins. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Tamura et al., 2011). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The analysis involved 143 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 12 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Each hydrophobin is identified by a GI number (with the exception of *L. bicolor* and *P. chrysosporium* which are identified by JGI protein ID's) and by the organism from which they were sequenced. *LbH* genes that group with saprotrophic fungal hydrophobins are marked by brown dots and *LbH* genes that group with mutualistic fungal hydrophobins are marked with green dots.

hydrophobins have most recently been implicated as a category of MiSSP during mutualistic interactions in the formation of cell:cell interfaces as well as in the protection from host defense responses (Duplessis et al., 2001, 2005; Mankel et al., 2002; Le Quéré et al., 2006; Rajashekar et al., 2007). As ECM fungi have evolved from saprotrophic ancestors (Hibbett and Matheny, 2009), the role of

some hydrophobins at the symbiotic interface is a new function likely to have evolved at the same time as the switch in lifestyle. Unfortunately little is known concerning which hydrophobins are expressed during symbiosis nor the role of these proteins have in mutualism. Given the availability of two draft genomes/sequences for the ECM fungus *L. bicolor* (strains S238N-H82 and 81306) and

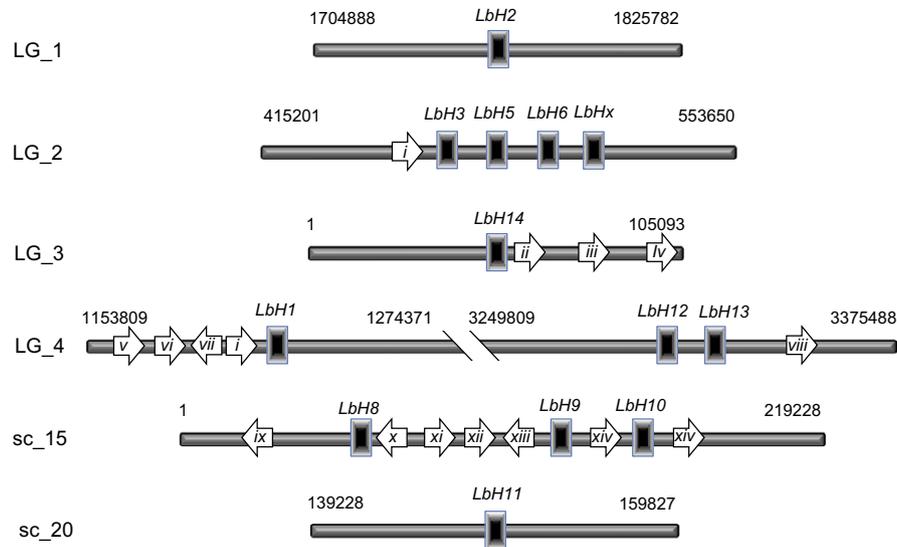


Fig. 3. Transposon clusters in *L. bicolor* S238N localize near paralogous genes. Graphical representation of the distribution of full length transposable elements within a 60 kb window upstream and downstream of each hydrophobin gene. The coordinates of each pseudochromosome are noted above each LG or scaffold. Transposable elements are as follows: (i) HMM_ReconFam_1523; (ii) MITE_X3; (iii) MITE_X2; (iv) T_SCF90_1; (v) HMM_Recon_Fam_1417; (vi) ReconRam_1725; (vii) FOT1-like; (viii) ReconFam_8213; (ix) ReconFam_1037; (x) ReconFam_30; (xi) ReconFam_2359; (xii) ReconFam_1049; (xiii) ReconFam_33; (xiv) ReconFam_641.

the range of host plants for this ECM fungus, we sought to address the first part of this question by conducting a genome-wide analysis of the hydrophobin gene repertoire in *L. bicolor* and to characterize their expression during the interaction of the fungus with different hosts. Our aim was to identify which hydrophobins of *L. bicolor* are important during the colonization process and to determine if these genes were different in character from other fungal hydrophobins.

4.1. Plant host and life stage alters regulation of hydrophobins

We found that a far greater number of hydrophobins were regulated during the interaction of hyphae with a host plant as opposed to the formation of a fruiting body (Fig. 4). As hydrophobins are regulated by changes in the external environment (e.g. during host colonization; Wessels, 1996) or to aid host colonization (Corvis et al., 2005, 2006, 2007; Qin et al., 2007; Zhao et al., 2007; Amanianda et al., 2009; Wang et al., 2010), it is possible that the large variety of hydrophobins encoded by *L. bicolor* strains are a necessary adaptation to aid in the colonization of different plant hosts. Therefore, if hydrophobins may act to protect invading hyphae from plant defenses, a logical extension to this idea would be that a host which creates an inhospitable environment for colonization (e.g. higher defense responses), would induce the expression of a wider variety of hydrophobins as opposed to a host that is easily colonized. We observed this correlation between hydrophobin expression and the number of roots colonized by the fungus (Fig. 4). We would suggest, therefore, based on what is currently known for hydrophobins interacting with host tissues, that hydrophobins are expressed at higher levels in less receptive hosts to provide a thicker layer around the fungal hyphae to protect it from plant based defenses or to hide hyphal surface antigens which would induce a higher level of defense by the plant. Given our results, it would be attractive to broadly conclude that hydrophobin expression varies inversely with the ease of host colonization. It is likely that the story is far more complicated, however, and this may not always be the case. While it has been found that hydrophobin expression correlates to the pathogenesis of animal pathogens, in plant pathogens this same link has not been absolutely correlated to the ability of different fun-

gal isolates to colonize plant tissues (Parta et al., 1994; Thau et al., 1994; Brasier et al., 1995; Bowden et al., 1996). For this reason, the screening of hydrophobin expression should be undertaken on a variety of different ECM plant hosts to determine if the relationship between ECM fungal colonization rate and hydrophobin expression holds true.

4.2. Hydrophobins of *L. bicolor* exhibit both canonical and novel characteristics

Hydrophobins of both *L. bicolor* strains carried the signature traits of class I hydrophobin genes with no chimeric class I/class II hydrophobins (Jensen et al., 2010). This is consistent with all other basidiomycetes which exclusively encode class I hydrophobins. The amino acid sequence of the *L. bicolor* hydrophobin genes are very conserved between the two strains separated both geographically and by evolutionary time. There are a number of differences in *L. bicolor* hydrophobins from both strains, as compared to classical hydrophobin genes, that may impact their role during interaction with plant hosts. Phylogenetic analysis of *L. bicolor* hydrophobins revealed what would appear to be the beginnings of divergence between the sequences of mutualistic and saprotrophic hydrophobins with a number of hydrophobins from *L. bicolor* and *P. involutus* grouping together and separately from saprotrophic hydrophobins (Fig. 2; highlighted in green). While some of these genes are differentially regulated both in fruiting bodies and mycorrhizal root tips (e.g. *LbH5*), suggesting that they have a role closer to saprotrophic hydrophobins, some hydrophobins appear to have developed (or are developing) a role in symbiosis as they are exclusively up-regulated during the formation of mycorrhizal root tips and not in the growth of free-living mycelium, during nutrient deprivation nor during the formation of the fruiting body (e.g. *LbH8*, 9/10; Fig. 4). A second difference of the hydrophobins encoded by *L. bicolor* is the higher degree of hydrophobicity of the core region between cysteine doublets as compared to other class I (e.g. SC3 from *Schizophyllum commune*) or class II hydrophobins (e.g. HFBI from *Trichoderma reesei*) (Kim et al., 2005; Jensen et al., 2010). Finally, a subset of the hydrophobins encoded by *L. bicolor* have an altered number of cysteine residues (Fig. 1). Additional cysteine residues have been identified

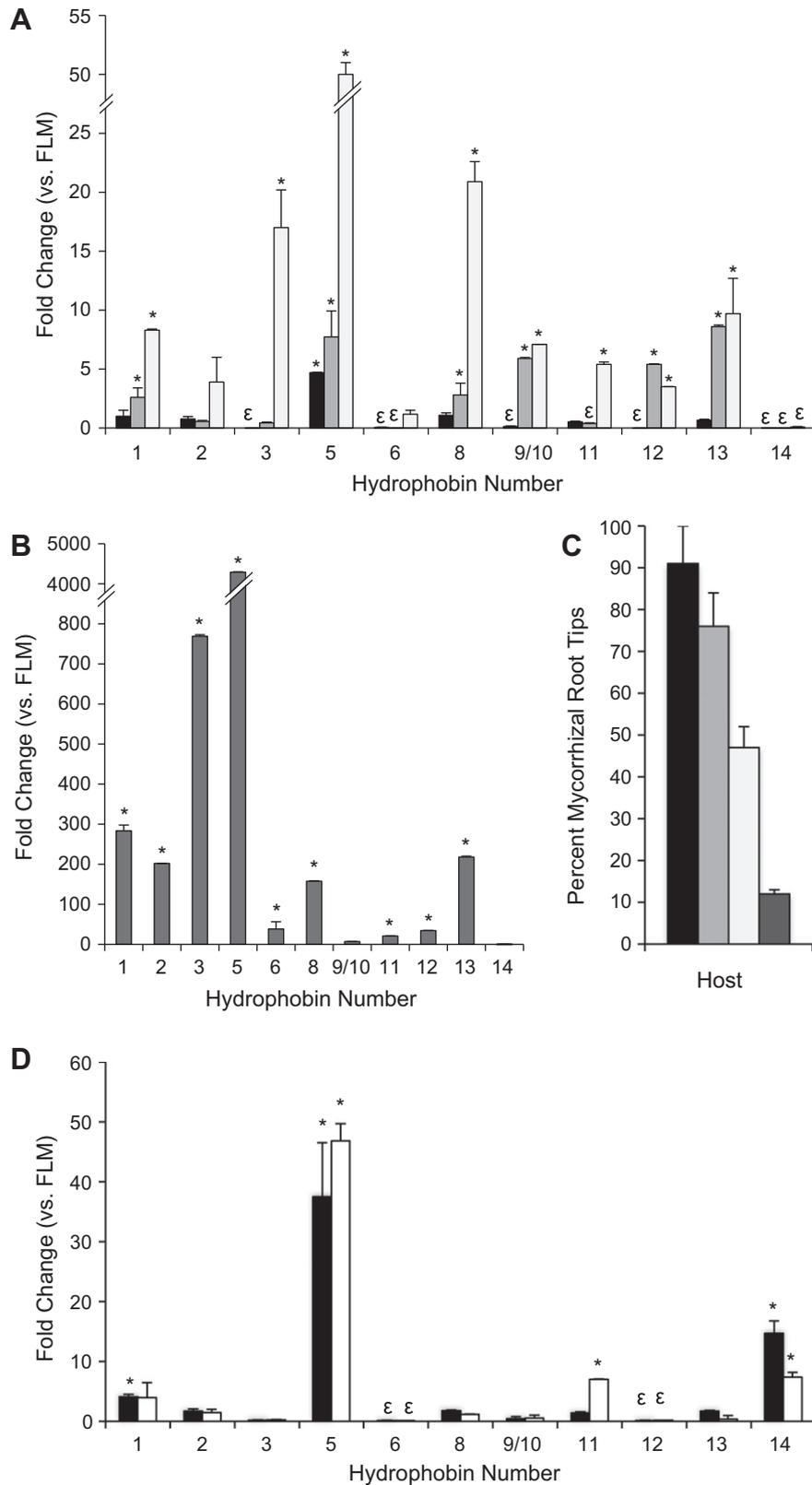


Fig. 4. Expression of *L. bicolor* hydrophobins varies with both morphogenic stage and host. (A) Expression of the hydrophobins in mycorrhizal root tips of Douglas fir (black bars), *P. trichocarpa* × *P. deltooides* clone 545 (light gray bars) and *P. trichocarpa* (white bars). (B) Expression of hydrophobins in mycorrhizal root tips of *P. deltooides* (dark gray bars). (C) Percent of lateral roots colonized by *L. bicolor* for Douglas fir (black bar), *P. trichocarpa* × *P. deltooides* clone 545 (light gray bar), *P. trichocarpa* (white bar) and *P. deltooides* (dark gray bar). (D) Expression of the hydrophobins in fruiting bodies of *L. bicolor* in association with Douglas fir (black bars) and *P. trichocarpa* (white bars). All expression values are expressed as fold difference from expression in free living mycelium (FLM) ± SE. * indicates significant up-regulation; ε indicates significant down-regulation ($p < 0.05$).

outside the canonical hydrophobin domains in a number of hydrophobins in *Aspergillus* species (Jensen et al., 2010), *Ustilago maydis* (Müller et al., 2008) and *Magnaporthe grisea* (Kershaw et al., 2005). As hydrophobins depend upon a specific cysteine spacing for proper folding and the maintenance of segregated charged and hydrophobic residues upon the protein surface (Sunde et al., 2008), these extra cysteines may alter protein folding, surface chemistry or may render them non-functional (Kershaw et al., 2005). Together, these data may indicate that a number of hydrophobins encoded by *L. bicolor* may be evolving roles specific to a mutualistic lifestyle.

4.3. Paralogous hydrophobins of *L. bicolor* are closely associated with transposable elements

The *L. bicolor* S238N-H82 genome encodes a high percentage of transposable elements (TEs) and TE fragments (21%; Martin et al., 2008), a number of which are transcriptionally active. Concurrent with the discovery of the large quantities of TEs was a higher representation of multigene families as compared to other fungal genomes. As TEs are associated with gene duplication and exon shuffling (Jiang et al., 2004; Morgante et al., 2005; Bennetzen, 2005), it was questioned at the time of the genome sequencing if the number of TEs, and of full length TEs especially, was tied to the expansion of these multigene families (Martin and Selosse, 2008). With the availability of a second *L. bicolor* genome we can now begin to answer this question.

The most obvious difference in between the hydrophobins encoded by *L. bicolor* S238N-H82 and *L. bicolor* 81306 is that the genome of S238N-H82 contains three paralogous genes (*LbH1*, *LbH9* and *LbH10*) not found in the 81306 genome. Due to the quality of the genome assembly, it is unlikely that these genes were missed do to sequencing gaps. When the localization of full length TEs (transposable elements with the highest likelihood of being active) was compared between all of the hydrophobins of the S238N-H82 genome, it was found that the paralogous genes *LbH1*, *LbH9* and *LbH10* resided beside, or within, the most dense and diverse islands of TEs (Fig. 3). In the current annotation of the 81306 genome, only TE fragments exist around the hydrophobin genes. As full length TEs are rapidly fragmented and lost (Bennetzen, 2005), and as TE islands are generally only found in sub-telomeric regions of the genome in other fungi (Stajich et al., 2010), the density of TEs around these paralogous genes would suggest that they were created by the movement of TEs. This would then support the theory that the higher than normal expansion of certain gene families in the *L. bicolor* S238N-H82 genome are linked to the numbers of TEs.

4.4. Conclusion and future perspectives

The hydrophobin genes of *L. bicolor* are numerous, varied in biochemical characteristics and are differentially expressed based on the fungal life stage and based on the host plant being colonized. Our results would suggest that there is a relationship between the expression of hydrophobins and the degree to which the fungus is able to colonize a host plant. Therefore, increased quantities of hydrophobins might be necessary to colonize host plants which mount a high level of defenses. The use of RNA silencing of the hydrophobins of *L. bicolor* could help determine some of the individual roles of hydrophobins as it is not possible based on the current analysis to identify whether any of the hydrophobins expressed during host colonization have unique roles necessary for mutualistic symbiotic exchanges. We would suggest rather that based on the number of hydrophobins expressed in colonized tissues, that as opposed to unique individual roles, it is likely that different combinations of hydrophobins are necessary for the fungus to colonize different hosts. The additive role of increasing

complexity in hydrophobin mixtures has been found to aid in attachment to cell wall materials and carbohydrates (Bell-Pederson et al., 1992; Carpenter et al., 1992; Lora et al., 1995). Therefore, while it is important to study and understand the role of each hydrophobin individually, our results would suggest that the best future avenue of study to truly understand the biological importance of the hydrophobin family will be to study the characteristics of different mixtures of hydrophobins and to determine their function as a whole.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2012.01.002.

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