Genomic and transcriptomic analysis of *Laccaria bicolor* CAZome reveals insights into polysaccharides remodelling during symbiosis establishment.

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KEY WORDS

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ABSTRACT

Ectomycorrhizal fungi, living in soil forests, are required microorganisms to sustain tree growth and productivity. The establishment of mutualistic interaction with roots to form ectomycorrhiza (ECM) is not well known at the molecular level. In particular, how fungal and plant cell walls are rearranged to establish a fully functional ectomycorrhiza is poorly understood. Nevertheless, it is likely that Carbohydrate Active enZymes (CAZyme) produced by the fungus participate in this process.

Genome-wide transcriptome profiling during ECM development was used to examine how the CAZome of *L. bicolor* is regulated during symbiosis establishment.

CAZymes active on fungal cell wall were upregulated during ECM development in particular after 4 weeks of contact when the hyphae are surrounding the root cells and start to colonize the apoplast. We demonstrated that one expansin-like protein, whose expression is specific to symbiotic tissues, localizes within fungal cell wall.

Whereas *L. bicolor* genome contained a constricted repertoire of CAZymes active on cellulose and hemicellulose, these CAZymes were expressed during the first steps of root cells colonization. *L. bicolor* retained the ability to use homogalacturonan, a pectin-derived substrate, as carbon source. CAZymes likely involved in pectin hydrolysis were mainly expressed at the stage of a fully mature ECM.

All together, our data suggest an active remodelling of fungal cell wall with a possible involvement of expansin during ECM development. By contrast, a soft remodelling of the plant cell wall likely occurs through the loosening of the cellulose microfibrils by AA9 or GH12 CAZymes and middle lamella smooth remodelling through pectin (homogalacturonan) hydrolysis likely by GH28, GH12 CAZymes.

1. INTRODUCTION

Ectomycorrhizal interactions (ECM) established between the root systems of perennial plants and hyphae from soil-borne fungi are ubiquitous in boreal and temperate forests. This symbiosis is a mutualistic interaction leading to fair nutrient exchanges. ECM fungi indeed obtain carbon (C) from the plant photosynthate and in return provide plant with some essential nutrients such as phosphate, nitrogen and potassium (Caseiri et al., 2013). These interactions are crucial for tree health and growth (biomass production) through increasing efficiency of nutrient uptake, modulating plant hormone signalling and finally increasing plant resistance to pests (Smith and Read, 2008). In addition, these interactions play a key role in nutrient recycling (Read and Perez-Moreno, 2003; Smith and Read, 2008).

The role of ECM fungi in soil C cycling may be multiple and likely opposite. They have been first considered as organisms leading to plant C sequestration into soils, as some studies estimate that 10-50% of the C-fixed by photosynthesis is allocated to ECM fungi (Simard SW, Jones MD, 2002). Indeed, radiocarbon analysis revealed that recently fixed carbon composed most of the biomass of ECM fungi (Henn and Chapela, 2001; Hobbie et al., 2002; Le Tacon et al., 2013; Treseder et al., 2006). Clemmensen et al. (2013) showed recently that in boreal forest islands, up to 70% of soil carbon was derived from roots and mycelium. They also showed that soils with the highest ECM fungi-derived biomass accumulate more rapidly C than soils containing saprotrophic fungi-derived biomass.

Secondly, in addition to saprotrophic fungi, ECM fungi have been considered as possible decomposers, thus participating to release of CO₂ to the atmosphere. Indeed, living ECM root tips display proteases, N-acetylglucosaminidase, glucuronidase, cellobiohydrolase, b-glucosidase, laccases activities (Burke et al., 2014; Courty et al., 2010), enzymes possibly active against cellulose, chitin and aromatic C compounds, respectively. In addition, several studies suggest that ECM fungi could grow from fungal or animal necromass to obtain C and N likely through high chitinase activity (Buée et al., 2007; Rajala et al., 2012). The role of ECM fungi as decomposers has been suggested to occur during the period of low C supply by the plant (Talbot et al., 2008) and ECM fungi are likely to better target soil nutrient (N and P) rich compounds, usually present in organic forms (Talbot and Treseder, 2010). Indeed, recent analysis showed that the ECM fungus *Paxillus involutus*, when growing on organic matter, is producing hydroxyl radicals through a Fenton system similar to the system used by wood-decomposing fungi to efficiently disrupt organic matter (Rineau et al., 2012). However, this degradative system is triggered by glucose, suggesting that this system is efficient to digest

organic matter but that the ECM fungi may not use the released carbon. This reinforces the idea that ECM fungi are degrading organic matter to access N and P rather than C, as the latter is delivered directly by the root cells (Talbot and Treseder, 2010). Whether or not ECM fungi retain saprotrophic growth ability for carbon sources under natural conditions is still under debate. Several studies demonstrated that the litter horizon of boreal and temperate forests is mainly inhabited with saprotrophic fungi, whereas the deeper horizons containing more decomposed litter and humus, are dominated by ECM fungi (Clemmensen et al., 2013; Lindahl et al., 2007). This vertical stratification suggests to the authors that it is unlikely that ECM fungi can grow saprotrophically on this low energetic value material (Baldrian, 2009).

Phylogenomic analysis of saprotrophic and ECM fungi from *Amanita* genus showed that apparition of ECM is associated with losses of cellulase genes playing a key role in saprotrophic growth (Wolfe et al., 2012). Recent comparative genomic analysis within Basidiomycota (containing saprotrophic and ECM fungi) highlight that ECM and brown rot fungi have a reduced or null set of certain plant Carbohydrate-Active enZymes (CAZymes). These losses or contraction are then correlated with the fungal way of life. In particular, Eastwood and colleagues (2011) highlight that ECM way of life was likely arising from brown-rot lifestyle. A similar correlation between fungal way of life and gene contraction has been shown regarding lignin-degradation enzymatic machinery (Floudas et al., 2012). Reduced numbers of these particular CAZymes, coupled with retention of the ability to degrade soil organic matter through oxidative mechanisms, suggest that ECM fungi are able to participate to the slow C cycle.

At the cellular level, morphogenesis of the ECM fungus-plant symbiotic interaction is well known (Brun et al., 1995; Horan et al., 1988; Kottke and Oberwinkler, 1987; Massicotte et al., 1987). Fungal hyphae form a mantle around lateral roots and penetrate within the apoplast of cortical root cells to form the Hartig net, the zone of nutrients and signalling compounds exchange. This intimate connection between root and fungal cells requires both fungal and plant cell wall remodelling to form the apoplastic symbiotic interface. On the plant side, elongation of epidermal cells and arrest of growth were observed (Peterson and Bonfante, 1994). Internal fungal hyphae are embedded in a matrix composed of polysaccharides, cystein-rich proteins and glycoproteins (Dexheimer and Pargney, 1991). Distribution of several fungal proteins such as hydrophobins, mannoproteins at the cell surface is changing during ECM establishment (Tagu and Martin, 1996). Symbiotic interactions rely on efficient communication between both partners and efficient nutrient exchanges through

the symbiotic interface. The plant cell wall is thus the place of the earliest communication event between plants and fungal symbionts. So far, it is not clear whether the fungal symbiont has a role in remodelling the plant cell wall during ECM development. Among the enzymes required for fungal and plant cell remodelling, CAZymes are likely the most important enzymes involved. Indeed, they are responsible for the biosynthesis, degradation and modification of oligo- and polysaccharides as well as of glycoconjugates. They are divided into four classes: Glycoside-Hydrolases (GH), Glycosyl Transferases (GT), Polysaccharide Lyases (PL) and Carbohydrate esterases (CE). Recently, several classes of Auxiliary Activities (AA) containing redox enzymes acting together with CAZymes to degrade lignocellulose material were added within the CAZy database (www.cazy.org, [Lombard et al., 2014]). All CAZymes and auxiliary activity enzymes can be attached to carbohydrate-binding modules (CBMs) that can promote enzymatic deconstruction of polysaccharide (Levasseur et al., 2014; Boraston et al., 2004; Hervé et al., 2010).

All together, previous analyses suggest that CAZymes may be crucial enzymes to explain ECM double way of life. Indeed, they may be involved throughout the continuum between saprotrophy and mutualism: from soil C-acquisition, fungal cell wall morphogenesis to plant cell wall remodelling for symbiosis establishment. To address the potential roles of fungal CAZymes within this continuum and their putative role in symbiosis establishment, we took advantage of the availability of the genome of the ECM fungus *Laccaria bicolor* (Basidiomycotina, Agaricomycotina, Agaricales, Hydangiaceae) (Martin et al., 2008) as well as other genomes within the Agaricomycotina sub-phylum (Floudas et al., 2012). Genome analyses already revealed that, compared to other fungi, *L. bicolor* possesses an enhanced repertoire for protein degradation but a reduced repertoire of CAZymes targeting cellulose and lignin (Martin et al., 2008). In this report, we combined genomic and transcriptomic analyses to provide a comprehensive view of the CAZome of *L. bicolor* and its regulation during symbiosis establishment. We compared the abundance and distribution of CAZyme families in *L. bicolor* to those found in other Basidiomycetes with various lifestyles. We further functionally analysed, by immunolocalization, one *L. bicolor* protein (expansin-like protein) whose expression is induced during ECM development.

2. MATERIALS AND METHODS

Detection and modular annotation of putative CAZymes in the genome of *Laccaria bicolor*

The search for catalytic modules specific to CAZymes (GHs, GTs, PLs and CEs) and their ancillary carbohydrate-binding modules (CBMs) in *Laccaria bicolor* was performed exactly as for the daily updates of the Carbohydrate-Active enZymes (CAZy) database (<http://www.cazy.org>) (Lombard et al., 2014). Briefly, the sequences of the proteins in CAZy are cut into their constitutive modules (catalytic modules, CBMs and other non-catalytic modules or domains of unknown function). Each is assigned to a class and family according to its similarity to manually created groups. The resulting fragments are assembled and formatted in sequence libraries for BLAST searches. Accordingly, each protein model from *Laccaria bicolor* was BLASTed against the libraries of over 200,000 individual modules using a database size parameter identical to that of the NCBI non-redundant database. All models that returned an e-value passing the 0.1 thresholds were automatically sorted to an intermediate report and manually analyzed. Manual analysis involved examination of the alignment of the model with the various members of each family (whether of catalytic or non-catalytic modules), with a search of the conserved signatures/motifs characteristic of each family.

Functional annotation of putative CAZymes

In the absence of experimentally assessed biochemical data, protein models were systematically compared against all models assigned to the different CAZyme families and specifically against the sequence of biochemically characterized enzymes. This strategy allows a straightforward activity prediction for protein models with strong similarity to experimentally characterized fungal enzymes. On the other hand, functional prediction is more difficult for families lacking biochemically characterized fungal representatives; these families often correspond to glycosyltransferases and transglycosidases. The accuracy of the functional predictions and therefore the functional descriptors therefore depend on the distance between the sequence of interest and its closest experimentally characterized counterpart.

Comparative study of CAZymes repertoire.

The CAZyme repertoire of *L. bicolor* was compared to those of other genome-sequenced fungi exhibiting a range of nutritional modes and mainly belonging to Basidiomycota phylum (Table S1). The last version of CAZyme database containing new families was used and consequently, the repertoire already published of several fungi may have slightly changed.

Phylogenetic analysis

Sequences significantly similar to the CBM1-bearing GH5 cellulase of *L. bicolor* were searched in the CAZy database. Only sequences with a complete GH5 module were selected.

Sequences were aligned using MUSCLE (Edgar, 2004) with default parameters. Sequences that were 100% identical were discarded to avoid uninformative redundancy. The multiple alignment was used as input for MrBayes (Ronquist et al., 2012) and RAxML (Stamatakis, 2014), to produce Bayesian and Maximum Likelihood phylogenies, respectively. MrBayes was run with 500,000 simulations with a mixture of models of evolution, an evaluation of the proportion of invariable sites and of the shape of the gamma distribution of evolutionary rates. RAxML was run with 1,000 bootstrap replicates, an estimation of the proportion of invariable sites and of the shape of the gamma distribution. The WAG model of evolution was selected for the ML phylogeny because it returned the highest posterior probability in the Bayesian analysis. The topology of the Bayesian tree was chosen as a reference and corresponding bootstrap values obtained from the ML tree were superimposed. The tree was annotated for protein module architecture using the online software iTOL (Letunic and Bork, 2007).

Biological material

The origine of *Laccaria bicolor* strains are described in Martin et al., 2008. We used the progenitor dikaryon S238N for ectomycorrhiza establishment and all expression data, whereas the haploid monokaryotic line S238N-H82 was sequenced and used for growth assays. *Laccaria bicolor* S238N-H82 was cultured in duplicate on Pachlewski medium for 17 days at 25°C to determine growth differences on 35 carbon sources (full list and dataset at www.fungal-growth.org) after which pictures were taken. No differences in diameter or morphology were observed between the duplicates.

Transcriptome analysis

For the mycorrhiza time –course experiment cuttings of *Populus trichocarpa* (clone INRA 101-74) were grown in pots containing TerraGreen® (an expanded clay soil conditioner, Brenntag Lorraine, Toul, France) mixed with fungal inoculum in a peat-vermiculite mix (4:1, v/v). After two, four, six and 12 weeks of contact mycorrhizal roots were harvested and immediately frozen in liquid nitrogen. RNA extraction, cDNA synthesis and array hybridizations were performed as described in Martin et al., 2008. Four biological repeats were performed.

Average expression levels were calculated for each gene from the independent probes on the array and were used for further analysis. Raw array data were filtered for non-specific probes (a probe was considered as non-specific if it shared more than 90% homology with a gene model other than the gene model it was made for) and renormalized using RMA and quantile normalization implemented in the ARRAYSTAR software (DNASTAR, Inc. Madison, WI, USA). A transcript was deemed expressed when its signal intensity was three-

fold higher than the mean signal-to-noise threshold (cut-off value) of the random oligonucleotide probes present on the array (50 to 100 arbitrary units). Negative expression values were set to 1 and the ratio is identical to the expression value. Log2-transformed data were calculated and were subjected to the CyberT statistical framework (<http://www.igb.uci.edu/servers/cybert/>) (Baldi and Hatfield, 2002). It employs statistical analyses based on regularized t-tests that use a Bayesian estimate of the variance among gene measurements within an experiment. Transcripts with a Bayesian P-value <0.05 and a more than three fold change in transcript level were considered as significantly differentially expressed. The expression data for all annotated CAZymes are summarized in Supplemental Table S4 and S5.

Quantitative RT-PCR

cDNA equivalent to 25 ng of totalRNA was used as template for each PCR reaction using SYBR[®] Green supermix (Biorad) with a final concentration of 1,6 mM of each primer. The thermal-cycling condition parameters, ran on the RotorGene System (Qiagen) were as follows: 95°C for 3 min; 40 cycles of 95°C for 15s, 60°C for 30 s followed by a melting curve. PCR amplifications were carried out on three biological replicates and included two distinct technical replicates. Transcript abundance was normalized using two constitutively expressed fungal genes. The ratios of expression between two conditions were calculated using Pfaffl et al. 2011.

Laser-scanning confocal microscopy.

Twelve-weeks old ECMs were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH 7) overnight at 4°C. Fixed tissues were washed in PBS buffer (1×) and embedded in 6% agarose (w/v). Sections of 30 µm were prepared using a VT1000S Leica vibratome (Leica Microsystems, Nanterre, France). For indirect immunofluorescent localization, a rabbit antibody against LbExp1 was generated against one peptide coupled to KLH (LASEDVGRIQVTWDYL) according to the manufacturer's procedure (Eurogentec, Seraing, Belgium). IgG-fraction and pre-immune serum were purified and desalted using MabTrap kit (GE Healthcare, Orsay, France). Immunolocalization was performed as previously described (Martin et al. 2008) Sections were incubated with IgG purified from pre-immune sera at the same concentrations for control observation. For co-localization study, the goat anti-rabbit IgG Alexa fluor 546 conjugate (1:400) was used as secondary antibody (Molecular Probes, Carlsbad, CA) in combination with Uvitex (0.1%). Sections were observed with a Radiance 2100 AGR3Q-BLD Rainbow microscope (Bio-Rad).

Immunogold microscopic analysis

Mycelium and mycorrhizal tips were prepared for transmission electron microscopy (TEM) by overnight fixation at 4°C in 2.5% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer pH 7.2 (Balestrini et al., 1996). After washing with the same buffer, osmium tetroxide (1% w/v) postfixation, ethanol dehydration, and ethanol/LR White (Sigma) infiltration, samples were embedded in LR White resin. Immunogold labelling was performed on thin sections (Balestrini et al., 1996) using the anti-LbExp1 antibody. Samples where the primary antibody was omitted were used as labelling-specificity controls.

3. RESULTS AND DISCUSSION

Annotation of the symbiont *Laccaria bicolor*'s CAZome reveals differences from CAZomes of Ascomycetes and other Basidiomycetes.

Secreted carbohydrate-degrading enzymes are crucial enzymes for fungal biology, both for fungal cell wall turnover and for degradation of external carbohydrate sources. Using the CAZY annotation system, CAZymes of *L. bicolor* S238N-H82 were identified, annotated (Table S2) and compared to those of Ascomycetes and Basidiomycetes of different lifestyles (from soil saprotrophic to pathogenic fungi including brown and white rot fungi) (Table S1). Our analysis first showed that the *L. bicolor* genome contains almost twice as many secreted carbohydrate-degrading enzymes (Glycoside-Hydrolases (GH), Polysaccharide Lyases (PL), and associated activities like Carbohydrate Esterases (CE)) than polysaccharide biosynthetic and modifying enzymes (mostly glycosyltransferases (GT) and transglycosidases of the GH class), a feature frequently observed in fungi due to their dependence on external carbohydrate sources.

Within the Agaricomycotina sub-phylum, *L. bicolor* shared with the brown-rot fungi *Coniophora puteana*, *Dacryopinax* sp., *Gloeophyllum trabeum*, the plant-pathogens *Heterobasidion annosum*, *Tremella mesenterica* Fries the smallest number of carbohydrate-binding domain (CBM) (Table 1). *L. bicolor* also shared with the brown-rot fungi *Serpula lacrymans*, *Dacryopinax* sp., the soil saprotroph *Agaricus bisporus*, the white-rot fungus *Phanerochaete chrysosporium* and the plant-pathogens *H. annosum*, *T. mesenterica* Fries the smallest number of GH (Table 1). The same feature was observed in the genomes of two biotrophic fungi interacting with plants *M. laricis-populina* and *U. maydis* and the Ascomycotina ECM fungus *Tuber melanosporum*. A reduced set of GH and CBM domains was thus supposed to be a hallmark of convergent evolution for fungi interacting with living plant cells (Veneault-Fourrey and Martin, 2010). Now, however, this assumption has to be revised, as more fungal genomes become available in particular the genomes of ericoid and

orchid mycorrhizal fungi which display the opposite trend (Martin, personal communication). Indeed, several brown-rot fungi such *C. putenea*, *Fomitopsis pinicola* displayed a high number of GH proteins and on the other hand white-rot fungus such as *Fomitiporia mediterranea* displayed a low number of CBM domains. We can rather suggest a continuum between these life-styles. These comparative genomic analyses of the main CAZyme classes are consistent with the recent assessment that the lines between brown rot and white-rot fungi are blurred, in particular regarding their lignin-degrading enzymes content (Riley et al., 2014).

Expression of CAZymes during ECM ontogenesis

CAZyme expression using microarrays were first analyzed in four different growth conditions (ECM root tips of poplar or Douglas fir; fruiting-body and free-living mycelium) and published in 2008 (Martin et al., 2008). This analysis revealed 121 CAZyme encoding genes significantly differentially regulated between free-living mycelium and ectomycorrhiza and/or fruiting-bodies (Martin et al., 2008).

We further characterized expression profile dynamics of *L. bicolor* CAZymes during ECM ontogenesis on poplar at different time points in greenhouse: 2, 4, 6 and 12 weeks after initial plantation of cuttings in pot containing fungal hyphae. Two weeks time-point corresponds to the initial contact between both partners and to mantle formation, 4 weeks to fungal colonisation of the first epidermal root cells, 6 weeks to fungal colonisation of cortical root cells and 12 weeks to the formation of Hartig net, where the interface of nutrient exchange takes place. Expression of 162 CAZyme genes was differentially regulated at the transcriptional level in at least one time point of the time course compared to free-living mycelium. Among them, 62% were upregulated after 4 and/or 6 weeks of initial plant-fungus contact. Interestingly, our transcriptomic analysis revealed that the vast majority of CAZymes are sequentially expressed during ECM development, suggesting a dynamic regulation over-time of these genes.

***Laccaria bicolor* CAZymes with possible activity on either fungal and/or plant cell wall**

The *L. bicolor* genome shared with the genomes of the mutualist *P. indica*, the brown-rot *Dacryopinax* sp. and the soil saprotroph *C. cinerea* the largest set of proteins of families GH5, GH16, CAZymes potentially active either on fungal or plant cell wall β -glucans (Fig.1; Fig. 2; Table S2).

L. bicolor presented an abundant set of GH16 enzymes with 31 members. Eighteen members were annotated as either candidate β -1,3-1,4-glucanase or β -1,3-glucan binding domain (Fig.1

and Table S2). Several observations suggest that fungal members of family GH16 enzymes are involved in the formation of the chitin- β -1,3-glucan complex, suggesting a role in the processing of fungal cell-wall polysaccharides (Klis et al., 2007). On the other hand, one report shows that in *Aspergillus fumigatus* one GPI-anchor endo- β -1,3-glucanase ENG2 (GH16 family) hydrolyzes β -1,3-glucans and carboxymethyl cellulose (Hartl et al., 2011). In addition, a patent describing the use of fungal GH16 polypeptides in combination with cellobiohydrolase and/or endoglucanase increases the rate of cellulose degradation (Corporation, 2013). These results show that GH16 members may act on either or both plant and fungal cell wall. Ten candidate β -1,3 or β -1,4-glucanases and/or lichenase were differentially expressed during ectomycorrhiza development with seven differentially expressed at 4 and 6 weeks of development (Fig. 3 Cluster D, Table S4). However, four of these GH16 candidates were also highly expressed in free-living mycelium (Table S4). In addition, *L. bicolor* genome encoded a large set of enzymes of GH5 family with eighteen out of the twenty-one GH5 members annotated as candidate β -glucosidase and exo β -1,3-glucanases (Table S2). Nine of them were significantly regulated during ECM development, eight at 4 and 6 weeks and one at 2 weeks (Fig. 3 cluster D and C, respectively; Table S4). Although not significantly regulated during ECM time-course, three were highly expressed at all time points, one at late time point (6 and 12 weeks) and two other at early time-points (2 and/or 4 weeks) (Table S5). Finding the exact substrate for these enzymes (GH16 and GH5) may help to assess whether these enzymes are required for degradation of fungal and/or plant cell wall. In particular, it would be useful to understand whether they are involved in saprotrophic growth of *L. bicolor* and/or are required for loosening plant/fungal cell wall in order to allow fungal colonization.

***Laccaria bicolor* expresses CAZymes active on its own cell wall during ECM development.**

CAZymes active on β -glucans

As mentioned in previous paragraph, *L. bicolor* presented an abundant set of GH16 enzymes with 31 members (Fig.1 and Table S2). Thirteen of them were annotated as β -1,6-glucan active enzyme (Table S2) and 6 of them were significantly regulated during ECM development. β -1,6-glucan is glucan specific to fungal cell wall. One GH16 of *L. bicolor* (294393), which is appended to a chitin-binding module of family CBM18 could be involved in the cross-linking of β -1,3,-glucan to other cell wall polysaccharides. This gene was upregulated in free-living mycelium, but it was also highly expressed at 4 and 6 weeks of

ectomycorrhiza development. Two members of GH16 encoding candidate β -1,6 glucanases were upregulated at early time-points of ECM development (after 2 and 4 weeks contact between roots and fungal hyphae)(Fig. 3 cluster C; Table S4) and three at intermediate (4 and 6 weeks) time-points (Fig.3 cluster D; Table S4). Although not significantly regulated during ECM development, two candidate β -1,6-glucan active enzyme (186913 and 253036) were highly expressed throughout ECM development (Table S5) and one (174712) at 4 weeks (Table S5).

In addition to GH16 family, *L. bicolor* genome contained ten candidate family GH30 β -1,6-glucanases. Together with the brown-rot fungus *F. pinicola*, *L. bicolor* genome displayed the highest number of GH30 found in fungal genomes yet (Fig. 1). Two β -1,6-glucanases GH30 (313498; 319085) were upregulated in mature ECM (Fig. 3 cluster B; Table S4) and one (303072) was highly expressed in free-living mycelium and another one (317861) throughout ECM development (Table S5).

L. bicolor also encoded a large set of enzymes of family GH5 with 21 members and only three annotated as candidate β -1,6- glucanase (Table S2). One out of the three candidate β -1,6-glucanase (305032) was upregulated after 4 and 6 weeks time point of mycorrhization (Fig. 3 cluster D; Table S4), whereas the two others (313918 and 298970) were not significantly regulated during ECM development but both displayed high expression level in free-living mycelium and at 2 and 4 weeks of ECM development (Table S5).

The *L. bicolor* genome encoded the largest number (8 members) of family GH72 cell-wall active enzymes among the compared fungi. These CAZymes are putative β -1,3-glucanosyltransglycosylase (EC 2.4.1.-) involved in fungal cell wall formation, maintenance and dynamics during different stages of the fungal life cycle (Balestrini et al., 2012; de Medina-Redondo et al., 2008; Gastebois et al., 2010; Ragni et al., 2007). Functionally characterized fungal GH72 enzymes catalyze the transglycosylation of β -1,3-glucans and play a crucial role in β -1,3-glucan cross-linking with other cell wall β -glucans (Mouyna, 2000). Five of the predicted GH72 enzymes were significantly regulated during ECM development in particular one (332893) at 2 and 12 weeks and four (166563; 187145; 310630; 143003) at 4 and 6 weeks (Fig. 3 cluster A and D; Table S4), suggesting an important role in symbiosis development, establishment.

CAZymes active on chitin

In addition to glucans, the fungal cell wall also contains chitin. The *L. bicolor* repertoire of enzymes for the metabolism of chitin was large with an expansion of chitin synthases of

family GT2 (Table S2) and chitin deacetylase of family CE4 (that transform chitin into chitosan) (Fig.1). This expansion of the chitin metabolism was also observed in the saprobe *Rhizopus oryzae* (Battaglia et al., 2011) and in the plant pathogenic fungus *Melampsora laricis-populina* (Duplessis et al., 2011).

Two distinct biological roles have been suggested for fungal chitin deacetylase (CE4). They may first be involved in fungal cell-wall synthesis as suggested by the localization of CE4 in *Absidia coerulea* cell wall (Gao et al., 1995) and the co-action of both CE4 and chitin synthase at the cell wall site in *Mucor rouxii* (Davis and Bartnicki-Garcia, 1984). More strikingly, chitin deacetylases are involved in plant-fungi interactions. It has been proposed that *in planta* infection structures from fungal pathogens deacetylate their chitin to chitosan in order (i) to protect fungal cell wall from the plant chitinase action and (ii) to avoid detection by the plant, as acetylated chitin is less potent than chitin to trigger plant defenses responses (Tsigos et al., 2000). The role(s) of these CE4 enzymes in *L. bicolor* is still unknown but according to their expression profiles, it is likely that some of them are involved in cell wall synthesis, whereas others are perhaps involved in fungal colonization without eliciting plant defense responses. Indeed, six CE4-encoding genes were upregulated at 4 and 6 weeks (Fig. 3 cluster D), whether three others were significantly upregulated at 2 and/or 4 weeks of ECM development (Fig.3 cluster C). This latter group of genes may be the better candidates to conceal chitin in the first steps of ECM establishment in order to avoid plant defense triggering.

The *L. bicolor* genome contained four candidate chitinases GH18 predicted to be secreted (Table S2). All these GH18 encoding genes were expressed at different time point of ECM development (Fig. 3; Table S4), suggesting a continuous degradation of chitin during ECM ontogenesis. These genes though upregulated during ECM development also displayed a high level of expression in free-living mycelium (Table S5). Thus, a potential role of these enzymes in the degradation of exogenous fungal cell wall should also be investigated, as it is known that ECM fungi may grow on dead fungal material during their saprotrophic way of life.

CAZymes active on α -glucans

Unlike the CAZymes active on β -glucans and chitin, the set of candidate enzymes acting on α -glucans in *L. bicolor* was very similar in abundance to that of other fungi. Indeed, as in most other fungal genomes, we found a single complete bimodular and bifunctional GH13/GT5 candidate α -1,3-glucan synthase along with four candidate α -1,3-glucanases of family GH71

and one candidate α -1,3-glucosidase of family GH63 (Table S2). The sole α -glucan synthase gene was upregulated at intermediate stages (4 and 6 weeks after contact) of ECM development. In *Cryptococcus neoformans*, α -1,3-glucan is involved in capsule formation and attachment (Reese and Doering, 2003). The plant pathogenic fungus *Magnaporthe oryzae* accumulates α -glucan on the surface of the cell wall on in-planta infection structures. This accumulation masks the chitin and the β -1,3-glucan, protecting the fungal cell wall, at least *in vitro*, against the action of plant chitinase and interferes with the plant immune system to delay pathogen-associated molecular patterns (PAMPS) detection (Fujikawa et al., 2012, 2009). It will be of great interest to verify whether *L. bicolor* is using α -glucan to either delay plant defense responses as per plant pathogenic fungi or to attach fungal hyphae to plant cell wall, thus preparing the construction of symbiotic interface.

In conclusion, most of the CAZymes that were significantly regulated during mycorrhizal ontogenesis are involved in the fungal cell wall processing. Altogether these results suggest an extensive cell wall remodelling when the fungus enters in contact with the host plant cells. This is consistent with the view of fungal cell wall as a highly dynamic structure, in which modifications of both composition and structure are a result of adaptation to changing environment (Latgé, 2007; Yin et al., 2008). This is also supported by a recent study analyzing the secretome of the free-living mycelium from *L. bicolor*. The *L. bicolor* secretome contains a high number of secreted CAZymes active on fungal cell wall (GH15, GH16, GH72) and chitin (GH18, CE4, CE9) (Vincent et al., 2009). The presence of these secreted enzymes, for instance two putative glucoamylases (GH15) and several putative α -glucosidases (GH31), may sustain the growth of *L. bicolor* on maltose (Fig. S1). In addition, this genetic repertoire may allow *L. bicolor* to recycle its own extracellular α -glucans in case of strong nutrient limitation leading to autophagy, supposed to occur at early time point of ECM establishment (Kim et al., 1999).

***Laccaria bicolor* genome contains a reduced set of lignocellulose active proteins.**

L. bicolor shared with all brown-rot fungi and the biotrophic fungus *M. laricis-populina* a reduced repertoire of CAZymes active on the plant cell wall (Fig. 2). The lowest set was found, as expected, in the animal pathogens *Cryptococcus neoformans* and *B. dendrobatidis*, the yeast *Pichia stipitis*, the plant-pathogenic fungus *Ustilago maydis* and finally the Ascomycete ECM fungus *T. melanosporum* (Fig.2), whereas white-rot fungi, soil saprotrophs and the endophyte *P. indica* displayed the most numerous enzyme sets.

Cellulose degradation

Degradation of cellulose is achieved by action of endo- β -1,4-glucanases (GH5; GH12), cellobiohydrolases or exo-1,4- β -glucanases (GH6 and GH7), which are assisted by β -glucosidases (GH3) that hydrolyze cellobiose and lytic polysaccharide mono-oxygenases (AA9, formerly GH61). Strikingly, no gene from families GH6 and GH7 were found in the *L. bicolor* genome, similarly to the symbiont fungus *T. melanosporum*, the brown-rot fungi *Dacryopinax* sp., *G. trabeum*, *F. pinicola*, the plant pathogens *U. maydis*, *T. mesenterica* and the animal pathogens *B. dendrobatidis* and *C. neoformans* (Fig. 2). Poor growth on cellulose but good growth on cellobiose (Fig. S1) can be explained by the absence of cellobiohydrolases (GH6, GH7) in the *L. bicolor* genome, while two β -glucosidases (GH3) were present. In addition a putative transporter of cellobiosides exists in the genome of *L. bicolor* (Martin et al., 2008). Interestingly, similar reductions and/or losses of cellulose acting CAZymes are shared with other ECM fungi like *T. melanosporum* or *Amanita bisporigera* (Martin et al., 2010; Nagendran et al., 2009), several obligate plant pathogens (Duplessis et al., 2011; Spanu et al., 2010), and the brown rot fungi *P. placenta* and *S. lacrymans*. However, we found three GH12 enzymes within *L. bicolor* genome annotated as candidate endoglucanase or xyloglucanase. These genes were regulated during ECM development with a higher expression at 2 weeks and in mature ECM for two of them (Fig. 3 Cluster A; Table S4). The one (385634) annotated only as a candidate endoglucanase was upregulated at 2 and 4 weeks of ECM development (Fig.3 Cluster C; Table S4). One GH12 endo-1,4- β -glucanase from *T. reesei* has been functionally characterized and promotes plant cell wall extension (Yuan, 2001). We can then speculate that these enzymes may be responsible for plant-cell remodelling at early fungal colonization.

Lytic polysaccharide mono-oxygenases (AA9, formerly GH61) frequently form a large multigene family in fungi, which feed on plant cell walls (Levasseur et al., 2013). Fungal AA9 enzymes enhance cellulase activity through oxidation of crystalline cellulose (Bauer et al., 2006; Beeson et al., 2012; Quinlan et al., 2011) and randomly cleave cellulose chains at the surface of the microfibrils, likely loosening the structure of cellulose microfibrils (Horn et al., 2012 ; Langston et al., 2011). *L. bicolor* genome contained eight AA9 genes, one of the lowest repertoires among fungi (Fig.1). Interestingly, three were upregulated in early (2 weeks) and mature (12 weeks) ECM (Fig.3 cluster A; Table S4), one was upregulated 4 weeks after contact with poplar roots (Fig. 3 cluster D; Table S4) and another one only in mature ECM (Fig. 3 cluster B; Table S4).

The extremely reduced set of cellulose-acting enzymes in *L. bicolor* is further reinforced by an almost total absence of cellulose-binding CBM1 modules. Indeed, *L. bicolor* contained only one CBM1 module fused to the only candidate endoglucanase of family GH5 predicted to be secreted (Table 1; Table S2). Interestingly, this gene was one of the most upregulated CAZymes in ECM compared to free-living mycelium (Martin *et al.*, 2008), but was not significantly regulated during ECM development (Fig. 3, Table S5). Cellulose-binding domain within the *Phytophthora* cellulose-binding elicitor lectin (CBEL) is a potent elicitor of plant defense responses (Gaulin *et al.*, 2006; Larroque *et al.*, 2013). It would be of interest to first check the ability of this domain to bind cellulose and then to investigate the role of such a domain in symbiosis development. We performed a phylogenetic analysis of the candidate cellulase that possesses the unique CBM1 cellulose-binding domain in *L. bicolor* (see methods). The phylogenetic tree showed that sequences similar to the *L. bicolor* GH5-CBM1 protein are clearly separated in two highly supported and monophyletic clades composed of Basidiomycetes and Ascomycetes, respectively. All Basidiomycete orthologs of *L. bicolor*'s protein possessed the same modular structure with an N-terminal signal peptide followed by a CBM1 module and ending with a GH5 module (Fig. 4). This suggests that they all derive from a common ancestral protein with the same architecture. The only exception here was the ortholog from *Schizophyllum commune* that does not contain a CBM1 module and that probably lost the module in a subsequent event. In Ascomycetes, in contrast, three different modular architectures were found, one similar to that of Basidiomycetes with a signal peptide, a CBM1 and a GH5 module (Fig. 4), another one with a signal peptide, followed by a GH5 module and with a C-terminally appended CBM1 (Fig. 4) and one last structure composed only of a signal peptide and a GH5 module. It is interesting to note that no single species featured a GH5 associated to a CBM1 with both the two possible architectures, suggesting that they are mutually exclusive. Ascomycete proteins that were most closely related to those of Basidiomycetes did not possess a CBM1 module. This observation and the presence of two types of CBM1-GH5 architectures collectively suggest that the CBM1 module may have been acquired and lost multiple times independently, possibly via exon shuffling.

Hemicellulose-active enzymes

Hemicelluloses mainly represented by xylan, xyloglucan and galactomannan cross-link cellulose microfibrils. Xylan degradation is achieved by a large variety of enzymes including endo- β -1,4-xylanases (GH10, GH11), α -L-arabinofuranosidase (GH3, GH10, GH43, GH51, GH54 and GH62), β -xylosidases (GH43 and GH3); acetylxyylan esterases (CE1-CE7).

We detected no hemicellulolytic enzymes in the genome of *L. bicolor* except for one putative acetylxyylan esterase CE5 (Fig. 2). *L. bicolor* did not have any endoxylanase encoding genes (GH10, GH11) in its genome, nor many genes encoding accessory enzymes involved in xylan degradation, explaining the poor growth on xylan and the inability of *L. bicolor* to use D-xylose as a carbon source. Reduced or loss of the ability to digest hemicellulose was shared with the ECM fungus *T. melanosporum*, the animal pathogens *B. dendrobatidis*, *C. neoformans*, the saprotrophs *T. mesenterica*, *P. stipitis* and *Phycomyces blakesleeana*. The only action on hemicellulose by *L. bicolor* may be due to its putative xyloglucanases of family GH12. Indeed two GH12 annotated as candidate endoglucanase or xyloglucanase were upregulated in early and mature symbiotic tissues as mentioned previously (Fig. 3 cluster A; Table S4).

Pectin-degrading enzymes

Pectin is a complex plant-cell wall polysaccharide composed mainly of homogalacturan (HG), rhamnogalactan I (RG-I), xylogalacturonan (XG) and rhamnogalacturonan II (RG-II). Efficient degradation of pectin is achieved with polygalacturonases and rhamnogalacturonases (GH28), pectin and pectate lyases (PL1, PL2, PL3), rhamnogalacturonan lyases (PL4, PL11), pectin methylesterases (CE8), pectin acetylerases (CE12, CE13) and rhamnogalacturonan acetylerases (CE12).

L. bicolor grows well on pectin (Fig. S1). The presence of six GH28 (candidate polygalacturonases) and two CE8 (candidate pectin methylesterases) are the best candidates to sustain this growth as *L. bicolor* genome had no pectin, pectate and rhamnogalacturonan lyases (PL1, PL2, PL3, PL4, PL9 or PL11), no α -L-arabinofuranosidases (GH43, GH51 or GH54) nor any α -rhamnosidases (GH78). The lack of GH43, GH51, GH54 and GH78 suggest that removal of L-rhamnose or L-arabinose residues is not likely the explanation for growth on pectin. It is thus predictable that the action of *L. bicolor* is limited to homogalacturonan through the action of six GH28 (candidate polygalacturonases) and two CE8 (candidate pectin methylesterases). These families were found in almost all fungi considered in this analysis. GH28 family was expanded in most genomes from brown-rot fungi. At least three members of *L. bicolor* GH28 were upregulated during ECM ontogenesis, two at early steps of ECM development and one at early and late time-points (Fig. 3 cluster C and A, respectively; Table S4). This suggests that these enzymes may be used to facilitate intercellular penetration of fungal hyphae through the pectin-rich middle lamella during ECM formation in living root cells by hydrolyzing homogalacturonan.

Family GH79

Among the most significant singularities in *L. bicolor*, we detected a particularly high number of candidate β -glucuronidase of family GH79 (EC 3.2.1.31) that could act on β -glycans either from fungal or plant origin (Fig. 2). GH79 family also includes enzymes involved in processing glycoproteins, inducing those found in plant cell walls (Eudes et al., 2008). This abundance (10 members) represents the highest total number found and is shared with two white-rot fungi, *Auricularia subglabra* and *Trametes versicolor*. We noticed that among the 10 GH79 members, four are specifically expanded in *L. bicolor* (Table S2). Seven of the 10 *L. bicolor* GH79 proteins bear a signal peptide and a GPI anchor modification site, as commonly observed among several characterized fungal cell-wall modifying β -glycosidases (Konishi et al., 2008). Characterized β -glucuronidases from *N. crassa* and *A. niger* hydrolyse β glucuronosyl and 4-O-methyl- β -glucuronosyl residues of arabinogalactan-protein (Konishi et al., 2008). Arabinogalactan proteins are plant glycoproteins, rich in hydroxyproline, serine, threonine and glycine and O-glycosylated carbohydrates such as galactan and arabinose (for review (Seifert and Roberts, 2007). Biological roles of plant arabinogalactan-proteins (AGP) are diverse but an increased number of studies highlight their role at the root surface to mediate root colonization by pathogenic and mutualistic microbes (Nguema-Ona et al., 2013) in plant-microbe interactions. Four *L. bicolor* GH79 members were upregulated between 4 and 6 weeks of ECM development (Fig. 3 cluster D; Table S4). It is thus tempting to speculate that these GH79 members of *L. bicolor*, by a putative role in hydrolyzing arabinogalactan-protein, may be involved in the communication between fungal cells and plant host cells.

In conclusion, *L. bicolor* has a restricted repertoire for CAZymes active on cellulose or hemicellulose, whereas it has retained the possibility to utilize pectin-derived material. Our analysis shows that the main plant cell wall-degrading enzymes present in *L. bicolor* genome are (i) endoglucanases, (ii) lytic polysaccharide mono-oxygenase, (iii) xyloglucanases, responsible for hydrolysis of xyloglucan, a compound contained in hemicellulose and (iv) polygalacturonase, responsible for pectin degradation. We showed a transcriptional regulation for these enzymes, with an up-regulation in mature symbiotic tissues in which the Hartig net is fully developed and/or at early time-point of ECM development during which fungal hyphae penetrate and colonize root tissues. All together, our results suggest that *L. bicolor* maintains and regulates enzymes allowing a “gentle” hydrolysis, remodelling the plant cell walls during fungal installation within the living root cells. However, our results also suggest that cellulose

remodelling is occurring during ECM development. This raises the question whether maintenance of cellulose integrity is important for the establishment of mutualistic interaction, as it is important for the outcome for plant-pathogenic microbe interaction.

***Laccaria bicolor* contains a large repertoire of expansin-like genes and one is a fungal cell wall protein.**

As a possible complement in the fungal and/or plant cell wall modification enzyme set, we observed an abundance of proteins distantly related to plant expansins. Their abundance seems to be a common feature of multicellular Basidiomycete fungi, as we find similar numbers in all Basidiomycetes except for *C. neoformans* (Table 1). Several studies showed that microbial expansins bind to plant cell polysaccharides and cause extension of cell walls without lytic action and without synergic effect on cellulases (Georgelis et al., 2014; Kerff et al., 2008; Saloheimo et al., 2002). In addition, swollenin silenced *Trichoderma asperellum* mutants are impaired in their ability to colonize roots (Brotman et al., 2008).

Eight expansin-related genes out of 12 in *L. bicolor* were regulated throughout the time-course (Fig. 3; Table S4). Six of them were upregulated during the intermediate time-point of ECM development (4 and 6 weeks after initial contact) (Fig. 3 cluster D; Table S4), whereas the other two were upregulated in both early and mature ECM (Fig. 3 cluster A; Table S4). Interestingly, though not regulated during ECM development, expansin-encoding genes may be highly expressed (Fig. 5A, Table S4, Table S5).

One expansin-like gene (JGI accession number 316565 called LbExp1) was first shown of the most highly induced CAZymes in ECM tissues (Martin *et al.*, 2008). This induction was confirmed using RT-qPCR (Fig. 5B). In addition, this gene is the sole expansin-gene which expression is specifically upregulated at 4 weeks after contact (Fig. 5A; table S4). To assess the role of LbExp1, we performed immunolocalization on mature ECM (12 weeks old) in order to ensure the presence of a Hartig net and a well-developed fungal mantle. LbExp1 was expressed in mantle and Hartig net (Fig. 6), in particular at the periphery of the hyphae suggesting fungal cell wall localization. Co-localization of LbExp1 and Uvitex, a fungal cell wall stain, confirms this cell wall localization (Fig. 6).

Transmission electronic microscopy coupled with immunogold localisation confirmed at a subcellular level that LbExp1 is a fungal cell wall protein of the Hartig net (Fig. 7 A, B, C) and mantle hyphae (Fig. 7D). Gold granules were also found associated to the dolipore septa (data not shown). Additionally to the fungal cell wall, gold granules were detected inside the hyphae, mainly in correspondence to vesicles present inside cytoplasmic multivesicular

bodies, which have been described as a part of the vesicular transport across cell wall in fungi (Casadevall et al., 2009). Only a weak labeling was observed on the fungal wall in the hyphae of the mycelium grown in pure culture (Fig. 7E). No immunogold labelling was detected in control experiments (Fig. 7F) where the primary anti-LbExp1 antibody was omitted. Some gold granules were also observed on the plant cell wall (Fig. 7B, D), suggesting that expansin proteins are present. However, immunolocalisation of LbExp1 protein showed that this protein is a fungal cell wall protein, suggesting a possible activity on fungal cell walls. In *A. nidulans*, a putative endoglucanase bearing an expansin-like protein EglD is localized to conidial cell wall and is likely involved in cell wall remodelling during germination (Bouzarelou et al., 2008). We can then hypothesize that LbExp1 is involved in fungal cell wall remodelling operating during the formation of symbiosis interface.

4. CONCLUSION

Our genomic and transcriptomic survey of *L. bicolor* CAZymes revealed the first insights of putative CAZymes involved in either fungal or plant cell wall remodelling to establish a functional symbiotic interface (Fig. 8). Our results suggest a highly active fungal cell wall remodelling process during ECM development especially after 4 weeks when fungal hyphae form the mantle and start to colonize epidermal root cells (Fig. 8). Indeed, the majority of genes that are significantly upregulated at this time point of mycorrhizal association are involved in the fungal cell wall synthesis or degradation. In addition, we showed that LbExp gene, which expression is specific to symbiotic tissues, is a fungal cell-wall protein. This results opens new perspectives to study the role(s) of fungal expansin, in particular in the field of plant-fungus interaction.

We also demonstrated that *L. bicolor* has a restricted repertoire for CAZymes active on cellulose or hemicellulose, whereas it has retained the possibility to utilize pectin-derived material, in particular likely homogalacturonan. Transcriptional regulation of these CAZyme families occurs during ECM ontogenesis. CAZymes active on hemicellulose are mainly expressed during mantle formation (first contact; 2 weeks); root cells penetration and colonisation of the first epidermal cells (4 weeks) (Fig. 8), whereas by contrast CAZymes active on pectin and cellulose are mainly expressed at 12 weeks. In addition, members of AA9, GH12, GH28 families are specifically expressed in symbiotic tissues. All together, our data suggest a subtle and gentle remodelling of the plant cell wall during ECM development. In particular, we can suggest a loosening of the cellulose microfibrils by AA9 and middle lamella smooth remodelling through pectin (homogalacturonan) hydrolysis by GH28 and

GH12.

Genomic and transcriptomic analysis are the first steps leading to functional analysis on a few CAZymes candidates either on their putative role for lignocellulosic material degradation or fungal material degradation. It will be also of great interest to assess what are the chemical modifications of cell walls occurring at different time point of ECM ontogenesis.

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[Figure Legends]

Figure 1. Distribution of fungal cell wall degrading enzymes in 26 fungal genomes.

The heatmap represents the abundance of each CAZyme family in each genome. The data were visualized and clustered using R (package HeatPlus). The hierarchical clustering was done by using an euclidean distance metric and average-linkage clustering method.

* indicates CAZymes with a possible effect on polysaccharides contained in both fungal and/or plant cell walls.

** indicates CAZymes with a possible effect on polysaccharides of fungal and/or bacterial origins.

Figure 2. Distribution of plant cell wall degrading enzymes in 26 fungal genomes.

The heatmap represents the abundance of each CAZyme family in each genome. The data were visualized and clustered using R (package HeatPlus). The hierarchical clustering was done by using an euclidean distance metric and average-linkage clustering method.

* indicates CAZymes with a possible effect on polysaccharides contained in both plant and/or fungal cell walls.

Figure 3. Hierarchical clustering tree view of 162 gene models coding for CAZymes from *Laccaria bicolor* with significant transcriptional regulation during a time-course for ECM ontogenesis (between 2, 4, 6 and 12 weeks).

Cluster **A** contains CAZymes with higher gene expression early in ECM development (2 or 4 weeks) and in mature ECM (12 weeks). Cluster **B** groups CAZymes with higher transcript levels in mature ECM (12 weeks), whereas Cluster **C** comprises CAZymes higher expressed in the first steps of ECM establishment (2 and/or 4 weeks). Cluster **D** contains CAZyme transcripts upregulated in intermediate time-points during ECM development (4 and 6 weeks). Transcripts significantly regulated (>3-fold; FDR, modified t-test <0.05) at minimum one of the time-points compared to free-living mycelium were included in the analysis. Relative expression indexes (REI) were calculated for the dataset. For each gene, a mean expression level was calculated from the four time-points, and the REI corresponds to the ratio between the expression level measured for a given time-point and the mean reference. Log2 transformed data were subjected to EPCLUST software (<http://www.bioinf.ebc.ee/EP/EP/EPCLUST/>) using correlation measure based distance (uncentered) and average linkage (UPGMA). Each gene is represented by a row of colored boxes (corresponding to REI values) and a single column represents each time-point.

Regulation levels range from pale to saturated colors (red for induction; green for repression). Black indicates no change in gene expression. EPCLUST (<http://ep.ebi.ac.uk/EP/EPCLUST/>) was used for the hierarchical clustering. For each cluster, a CAZyme gene list reads from left to right then up to down. Protein ID and CAZyme family are given for each gene and putative activities are reported according to the following color code: red (CAZymes active on fungal cell wall), green (CAZymes with candidate activity on plant cell wall), blue (activity on either fungal or plant cell wall).

Figure 4. Phylogenetic tree of CBM1-bearing GH5 proteins similar to *L. bicolor* candidate endoglucanase. The tree topology is shown according to the Bayesian phylogeny performed with 500,000 simulations using MrBayes. Gray circles on the branches directly connecting the considered node represent posterior probabilities with values >0.8. Corresponding support values obtained with a Maximum Likelihood phylogenetic tree performed with 1,000 bootstrap replicates using RAxML are indicated. Modular structures associated to the proteins present on this tree are represented on the right panel. Proteins that possess a CBM1 module appended to the GH5 module are divided in three monophyletic groups labelled A, B and C. List of species abbreviations is presented in supplementary table S3.

Figure 5. Expression of the 12 *Laccaria bicolor* expansin-encoding genes. (A) Heat-map displaying mean expression values throughout ectomycorrhizal time-course (2, 4, 6 and 12 weeks) and in free-living mycelium (FLM). * indicate genes which expression are differentially expressed in at least one condition. Other genes are not significantly differentially expressed. (B) Validation by quantitative RT-PCR of the over-expression in ECM compared with FLM of the expansin 316565 gene.

Figure 6. *L. bicolor* expansin-like protein co-localises with fungal cell walls within ECM structures.

(a) laser-scanning confocal microscopy image of a transverse section of 12 weeks-old *L. bicolor* - *P. trichocarpa* ectomycorrhizae
 (b) staining of fungal cell walls using Uvitex (green).
 (c) indirect immunolocalization of the *L. bicolor* expansin-like protein (red)

(d) Photoshop-merged image of b and c showing a colocalization of the *L. bicolor* expansin-like protein and Uvitex. m = mantle, hn = Hartig net, ec = epidermal root cell Scale bar, 5µm.

Figure 7. Immunogold-TEM localization of LbEXP on thin sections from *Laccaria bicolor*-poplar ectomycorrhizae (A, B, C, D) and *Laccaria* free-living mycelium (E).

(A, B). Using the anti-EXP, labelling was observed on the cell wall of mantle-forming hyphae. Arrows point to LbEXP-associated gold granules localized on the fungal cell wall. Scale bars: 0,3 µm and 0,35 µm for A and B respectively.

(C.) Magnification of a mantle region. Gold granules were found in association with vesicles present inside multivesicular bodies, additionally than on the fungal cell walls. Scale bar: 0,2 µm.

(D.) Labelling on the cell wall of hyphae progressing between root cells in the Hartig net region. **Inset**, magnification of labelled fungal cell walls. Scale bar: 0,8 µm and 0,3 µm for D and the inset respectively.

(E.) Only a few gold granules (arrows) were present on the walls of hyphae grown in pure culture. Scale Bar: 0,35 µm.

(F.) An ectomycorrhizal control section where the primary anti-Lb-Exp316565 antibody was omitted. Scale bar: 0,3 µm.

H, host cells; F, fungus; M, mitochondria; MVB, multivesicular bodies; S, septum; W, fungal cell wall. Asterisks indicate the plant cell wall.

Figure 8. Expression of *Laccaria bicolor* genes encoding CAZymes during ectomycorrhiza formation with poplar roots (2, 4, 6 and 12 weeks after contact).

A. Laser-scanning confocal microscopy image of transverse section of *Populus* roots 2, 4, 6 and 12 weeks after contact with *L. bicolor* hyphae. Plant root cells are counterstained with propidium iodide and fungal cell wall are revealed using Uvitex. Photoshop-merged images. m = mantle, hn = Hartig net, ec = epidermal root cell, cc = cortical cell Scale bar, 10µm

B. Expression profiles correspond to the different clusters identified in figure 4 (Clusters A to D)

C. Tendency for gene expression regulation of CAZymes targeting the different carbohydrates from cell-wall either from fungal or plant origin. CAZyme families within brackets indicate a family containing at least one member, which display specific expression to symbiotic tissues (i.e. which are not expressed in free-living mycelium).

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Table 1

Table 1. Distribution of the CAZyme modules in the gene repertoire of sequenced fungi with different life-styles..

GH, glycoside hydrolase; GT, glycosyl transferase; PL, polysaccharide lyases; CE, carbohydrate esterases; CBM: carbohydrate-binding module; EXP, plant-expansin-like

Phylum	Subphylum	Species	Way of life	GH	GT	PL	CE	CBM	EXP	TOTAL
Basidiomycota	Agaricomycotina	<i>Agaricus bisporus</i>	Soil Saprotroph	173	51	9	33	38	7	311
		<i>Coprinopsis cinerea</i>	Soil Saprotroph	185	64	13	50	89	15	416
		<i>Laccaria bicolor</i>	Mutualist	170	87	7	20	31	12	327
		<i>Schizophyllum commune</i>	White-rot fungus	226	70	16	40	43	23	418
		<i>Auricularia subglabra</i>	White-rot fungus	366	73	20	44	96	23	622
		<i>Serpula lacrymans</i>	Brown-rot fungus	164	62	6	13	43	15	303
		<i>Coniophora puteana</i>	Brown-rot fungus	243	55	3	20	31	19	371
		<i>Punctularia strigosozonata</i>	White-rot fungus	225	71	12	30	57	10	405
		<i>Dacryopinax</i> sp. DJM 731 SSP1	Brown-rot fungus	154	78	3	18	14	6	273
		<i>Gloeophyllum trabeum</i>	Brown-rot fungus	195	63	9	17	19	13	316
		<i>Fomitiporia mediterranea</i>	White-rot fungus	197	72	6	17	26	11	329
		<i>Fomitopsis pinicola</i>	Brown-rot fungus	197	71	3	21	41	16	349
		<i>Phanerochaete chrysosporium</i>	White-rot fungus	178	64	4	15	47	11	319
		<i>Trametes versicolor</i>	White-rot fungus	221	82	9	19	49	12	392
		<i>Heterobasidion annosum</i>	Plant pathogen	179	62	9	21	36	12	319
		<i>Piriformospora indica</i>	Mutualist	195	61	14	42	101	6	419
		<i>Cryptococcus neoformans</i>	Animal Pathogen	83	61	3	6	12	6	171
		<i>Tremella mesenterica</i> Fries	Plant pathogen	72	69	2	12	14	10	179
	Pucciniomycotina	<i>Melampsora laricis populina</i>	Plant pathogen	170	75	6	35	7	9	302
	Ustilaginomycotina	<i>Ustilago maydis</i>	Plant pathogen	101	56	1	13	6	10	187
Ascomycota	Pezizomycotina	<i>Aspergillus nidulans</i>	Soil Saprotroph	264	92	22	28	48	1	455
		<i>Trichoderma reesei</i>	Soil Saprotroph	199	92	5	16	54	4	370
		<i>Tuber melanosporum</i>	Mutualist	92	96	3	12	24	0	227
	Saccharomycotina	<i>Pichia stipitis</i>	Soil Saprotroph	54		0	3	7	0	64
Mucoromycotina	Mucoromycotina	<i>Phycomyces blakesleeanus</i>		80		4	23	15	11	133
Chytridiomycota	na	<i>Batrachochytrium dendrobatidis</i>		46		4	29	102	1	182

Figure 1

Fungal species

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 6

Figure 7

Figure 8

Figure 8.