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The ectomycorrhizal basidiomycete *Laccaria bicolor* releases a secreted endocellulase that plays a key role in symbiosis development

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Summary

- In ectomycorrhizae, root ingress and colonization of the apoplast by colonizing hyphae is thought to mainly rely on the mechanical force that results from hyphal tip growth, but this could be enhanced by secretion of cell wall degrading enzymes, which have not been identified yet. The sole cellulose-binding module (CBM1) encoded in the genome of the ectomycorrhizal *Laccaria bicolor* is linked to a glycoside hydrolase family 5 (GH5) endoglucanase.
- Here, we characterize the expression of *LbGH5-CBM1* gene and biochemical properties of its protein product. We show that *LbGH5-CBM1* expression is substantially induced in ectomycorrhizal poplar roots and RNAi mutants with a decreased *LbGH5-CBM1* expression have a lower ability to form ectomycorrhizae, suggesting a key role in symbiosis development.
- The recombinant LbGH5-CBM1 displays its highest activity towards cellulose, including poplar cellulose, and galactomannans, but no activity toward *L. bicolor* cell walls. *In situ* localization of LbGH5-CBM1 in ectomycorrhizae by immunofluorescence confocal microscopy reveals that the endocellulase accumulates at the periphery of hyphae forming the Hartig net and the mantle.
- Our data suggest that the symbiosis-induced endoglucanase LbGH5-CBM1 is an enzymatic effector involved in cell wall remodeling during formation of the Hartig net and is an important determinant for successful symbiotic colonization.

Introduction

In boreal and temperate forests, trees rely on the ectomycorrhizal symbiosis to acquire the scarce nutrients available in soils (Read *et al.*, 2004; Smith & Read, 2008; van der Heijden *et al.*, 2015). Ectomycorrhizal fungi establish a mutualistic association with host rootlets (Brundrett, 2002; Peterson & Massicotte, 2004) leading to dramatic morphogenetic changes in both symbiotic partners. After contacting the cortical cell surface of short roots, hyphae differentiate a fully developed pseudoparenchymatous sheath enclosing the root apex (Massicotte *et al*., 1989). Then, hyphae penetrate the root intercellularly after a loose hyphal weft has been formed between two rhizodermal cells to differentiate an intraradicular hyphal network, so-called the Hartig net (Balestrini & Kottke, 2017). The development of the Hartig net is initiated from the innermost layer of a fully differentiated mantle and radial penetration of finger-like hyphae takes place in a broad lobed hyphal front (Balestrini & Kottke, 2017). This channel-like hyphal structure is the most prominent feature of ectomycorrhizae. A common apoplast, so-called the symbiotic interface, resulting from the fusion of fungal and plant cell wall polysaccharides and proteins differentiates between plant cells and fungal hyphae favoring the bi-directional translocation of solutes (Smith & Read, 2008). Formation of the Hartig net leads to remodeling of the hyphae and cortical cell surface (Balestrini & Kottke, 2017). Although Hartig net development does not produce substantial qualitative change in cell wall composition, subtle alterations, such as localized loosening and swelling and redistribution of un-esterified pectins, are accompanying the hyphal ingression (Balestrini *et al*., 1996; Balestrini & Bonfante, 2014). In addition, immuno-cytochemical microscopy has revealed changes in the spatial distribution of cell wall proteins, such as hydrophobins and symbiosis-regulated acidic polypeptides of 32 kDa, in the *Pisolithus microcarpus-Eucalyptus globulus* association (Laurent *et al*., 1999; Tagu *et al*., 2001).

The root ingress and colonization of the host apoplast, e.g. middle lamella, by the colonizing hyphae is thought to mainly rely on the mechanical force that results from hyphal tip growth (Peterson & Massicotte, 2004). It has also been proposed that auxins released by the colonizing hyphae could promote root cell wall loosening (Gay *et al.*, 1994a,b) to ease the fungal penetration. Mutants of *Hebeloma cylindrosporum* over-producing auxins differentiate a multiseriate Hartig net (Gay *et al.*, 1994). Hyphal

penetration could also be enhanced by the secretion of fungal plant cell wall degrading enzymes (PCWDE), such as symbiosis-upregulated endoglucanases and polygalacturonases (Peterson & Massicotte, 2004; Martin *et al*., 2008; Veneault-Fourrey *et al*., 2014). As of today, no experimental data have been produced to support either one of the above mentioned hypotheses.

Cellulose being the major constituent of plant cell walls, its complete breakdown by fungal enzymes would likely involve at least three types of hydrolytic enzymes, endo-1,4-β-glucanase (EC 3.2.1.4) cleaving internal β-1,4-glycosidic bonds, cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21), releasing mainly cellobiose and glucose, respectively. It should be keep in mind that the genome of *the* ectomycorrhizal *L. bicolor* (Martin *et al.*, 2008), *T. melanosporum* (Martin *et al.*, 2010) and other ectomycorrhizal basidiomycetes sequenced so far (Kohler *et al*., 2015) have no cellobiohydrolases from family GH6 or GH7, no endoglucanase GH45 acting on cellulose and no or only one carbohydrate-binding module 1 (CBM1) involved in cellulose binding. Interestingly, the sole cellulose-binding module CBM1 found in the predicted proteome of *L. bicolor* is linked to a glycoside hydrolase of the family GH5 with a predicted secretion signal, named LbGH5-CBM1 (Martin *et al.*, 2008) and the corresponding transcript is upregulated during poplar ectomycorrhiza development (Martin *et al.*, 2008; Veneault-Fourrey *et al.*, 2014). The orthologous gene of LbGH5-CBM1 in the ectomycorrhizal Périgord Black Truffle (*Tuber melanosporum* Vittad) is also highly upregulated during symbiosis development (Martin *et al.*, 2010). The observed symbiosis upregulation of the *GH5-CBM1* gene in both *L. bicolor* and *T. melanosporum* and the absence of other secreted enzymes with a CBM1 suggest that the encoded enzyme may play a role in symbiosis development, i.e. host cell wall loosening, rather than participating to the breakdown of cellulose from soil organic matter.

In this study, we confirmed the role of the *LbGH5-CBM1* gene during symbiosis development by confirming its increased expression in ectomycorrhizae and showing that RNAi-mutants of *L. bicolor* have a lower ability to form ectomycorrhizae. We then purified the recombinant LbGH5-CBM1 and showed that the enzyme is a secreted endoglucanase able to cleave cellulose from poplar roots. By indirect immunofluorescence confocal microscopy, we also found that the enzyme accumulates

at the periphery of hyphae in the Hartig net and mantle. Our findings suggest the symbiosis-induced LbGH5-CBM1 endoglucanase plays a key role during symbiosis formation.

Material and methods

Biological material and growth conditions

Mycelial cultures of *L. bicolor* (Maire) P.D. Orton, strain S238N (from the Tree-Microbe Interactions Department Collection, INRA-Nancy, France**)** were grown on Pachlewski agar medium at 20°C in the dark and subcultured every month (Felten *et al.*, 2009). For the current experiments, the free-living mycelium was grown on modified Pachlewski agar medium at 20°C in the dark with cellophane placed on the surface for 10 days (Felten *et al.*, 2009) before mycelia were harvested for mycorrhizal inoculation or further enzymatic and expression analyses.

To assess the effect of increasing concentration of glucose and cellulose on the *LbGH5-CBM1* expression, 10-day-old mycelium was cultured in 100 ml modified low-glucose Pachlewski medium containing glucose (0.1% w/v, 0.05% w/v, or 0.01% w/v) or 0.01% (w/v) glucose with 0.25% (w/v) α-cellulose (C8002, Sigma, France) in 250 ml E-flasks at 20°C in the dark on a shaker (200 rpm) for seven days, and then harvested by filtration using a Büchner funnel under vacuum. After washing twice with Milli-Q water, the mycelium was harvested, frozen in liquid N_2 and stored at -80°C for RNA extraction (Dietz *et al.*, 2011).

The hybrid grey poplar (*Populus tremula* x *Populus alba*, INRA clone 717-1-B4) was used for *in vitro* mycorrhizal inoculation. Two-cm-long poplar cuttings were rooted on solid Murashige and Skoog (MS) medium (Felten *et al.*, 2009) for 3 weeks, and the fungal colonies of *L. bicolor* strain S238N (or transformed RNAi-silencing lines) were grown on Pachlewski agar medium in the dark with cellophane placed on the surface for 10 days. The membrane with *L. bicolor* mycelium and poplar seedlings with one or two main roots were transfered on the surface of a low-glucose Pachlewski agar medium (0.1% glucose) containing 0.1% MES (M8250, Sigma, France), and grown between two cellophane membranes. Plantlets were grown in a controlled environment growth room with 16 h photoperiod (22°C/18°C, day/night), 50–60% relative humidity and 400 µmol

m^{-2} s^{-1} photosynthetic photon flux density. Mycorrhizal roots were sampled at three weeks after contact, together with non-mycorrhizal plantlets grown for the same period of time, snap frozen in liquid N_2 and stored at -80°C until further analyses. For each sampling, five replicates were analyzed. Each replicate consisted of one dish containing three plantlets; 25 to 50 mycorrhizal root tips from these three plantlets were pooled and represent a single replicate.

Analysis of the LbGH5-CBM1 gene

The haploid genome from *L. bicolor* S238N-H82 was sequenced and annotated as described in Martin *et al.* (2008) (http://genome.jgi.doe.gov/Lacbi2/Lacbi2.home.html, RefSeq: NZ_ABFE00000000.1). Carbohydrate-active enzymes were annotated as described in Martin *et al.* (2008) by using the CAZy database (http://www.cazy.org). An endoglucanase GH5_5 with a CBM1 domain gene, named *LbGH5-CBM1*, was identified by automatic and manual annotation (Joint Genome Institute (JGI) accession number 319772, GenBank accession number XM_001879394.1). The nucleotide sequence of the *LbGH5-CBM1* gene was analyzed and compared to sequence databases by using available online tools (http://www.ncbi.nlm.nih.gov/, http://www.expasy.org). The likely sub-cellular localization of *LbGH5-CBM1* was determined using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The NetOGlyc 4.0 Server was used to predict potential *N*- and *O*-glycosylation sites (http://www.cbs.dtu.dk/services/NetOGlyc/).

Quantitative RT-PCR

Free-living mycelium (200 mg) and at least 15 mycorrhizal and non-mycorrhizal lateral rootlets from five different root systems of *P. tremula* x *P. alba* clone 717-1B4 colonized by *L. bicolor* were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the RNeasy Plant kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions with the addition of polyethylene glycol 8000 to RLC buffer (25 mg ml^{-1}). To avoid DNA contamination, a DNA digestion step was performed on-column with DNAse I (Qiagen, France). RNA quality was checked by using Experion HighSens capillary gels (BioRad, Marnes-la-Coquette, France). Synthesis of cDNA from 1µg of total RNA was performed using the iScript kit

(BioRad, France) according to the manufacturer's instructions. All primers were ordered from Eurogentec (Angers, France) and PCR amplification was performed using *Taq* DNA Polymerase (Thermo Fisher Scientific, France) according to the manufacturer's instructions and optimized according to each primer pairing. All primers were designed using *L. bicolor* genome version 2.0 (http://genome.jgi-psf.org/; Martin *et al*., 2008). Real-time PCR analyses were performed using the Fast SYBR Green Master Mix (Applied Biosystems, France) with a final concentration of 0.3 µM of each primer following the manufacturer's instructions. The thermal-cycling condition parameters of the StepOnePlus System qPCR apparatus (Applied Biosystems, France) were as follows: 95°C for 3 min; 40 cycles of 95°C for 15 s, 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 four constitutively expressed *L. bicolor* genes coding for a histone H4 (JGI ID# 319764), ubiquitin (JGI ID# 446085), a heat shock protein HSP70 (JGI ID 609242) and a mitochondrial substrate carrier protein (JGI ID# 611151). Primers sequences are given in Supporting Information Table S1 and were designed using available online tools (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Transcript abundance was quantified using the standard curve method of quantification (based on ΔΔCt calculations), as previously described by Pfaffl (2001).

Generation of L. bicolor *RNAi lines*

Transformation of *L. bicolor* S238N was performed using the RNAi/*Agrobacterium*-mediated transformation (AMT) vector for intron hairpin RNA (ihpRNA) expression, and transformation of *L. bicolor* vegetative mycelium used the pHg/pSILBAg vector system as described in Kemppainen and Pardo (2010) using the cDNA fragments of *LbGH5-CBM1*. To generate the mock transformant strains, *L.bicolor* S238N wild type was Agro-transformed with pHg/pSILBA*γ*. This mock vector carried a full-length, *Sac*I-linearized, empty pSILBA*γ* cloning vector in T-DNA of pHg. Three randomly selected pHg/pSg *LbGH5 L. bicolor* transgenic strains (*A4, G4 and H4*) and the strains transformed using the empty vector (lac ev7 and lac ev9) were used in this study. Three *L. bicolor* transformants with the *LbGH5-CBM1* RNAi construct and two mock transformants were screened for transgene expression by SYBR Green qRT-PCR assay.

Relative transcript abundance of *LbGH5-CBM1* and reference genes in the wild-type, empty vector and RNAi strains was quantified in *L. bicolor* mycelia grown on solid Pachlewski medium at 20 °C for 3 weeks and in poplar ectomycorrhizae ($n = 3 \pm$ SE). Transcript abundance of *LbGH5-CBM1* in free-living mycelium from the empty vector and the RNAi strains was close to the background expression values. As a result, no significant differences can be observed compared to the WT free-living mycelium (Supporting Information Fig. S3).

Yeast secretion trap assay

To construct a Gateway compatible vector for yeast secretion assay (Plett *et al.*, 2017), the nucleotide sequence corresponding to the signal peptide of LbGH5-CBM1 and the sequence of LbGH5-CBM1 without the native signal peptide were cloned into the pSMASH vector fused to the N-terminus of the yeast invertase (*suc2*) gene lacking its native signal peptide sequence using the Gateway method (Thermo Fisher Scientific, France) according to Lee *et al*., (2006). Secretion of the yeast invertase with LbGH5-CBM1 signal peptide is detected by yeast growth on the sucrose-selection medium.

Recombinant enzyme production and purification

The methylotrophic yeast *Pichia. pastoris* metabolizes methanol as its sole carbon source using alcohol oxidase (AOX). *P. pastoris* expression vector pPICZaA contains the AOX1 promoter for producing heterologous proteins (Ellis *et al*., 1985, Tschopp *et al*., 1987, Koutz *et al*., 1989) and a $(His)_6$ tag located at the C-terminus for purification. *P. pastoris* strain X-33 was purchased from Invitrogen (Cergy-Pontoise, France). The nucleotide sequences of *LbGH5-CBM1*, *LbGH5* and *CBM1* (Figure S1) were codon optimized for expression in *P. pastoris* and synthesized by Genscript (NJ, USA). They were then inserted into the expression vector pPICZaA in frame with the yeast α-factor secretion peptide at the N-terminus and the $(His)_6$ tag at the C-terminus, and under the control of the *AOX1* promoter. The expression protocol is described in Couturier *et al.* (2011). The large scale (2.4 L) production of each protein sequence was performed in 500 ml non-baffled flasks, each containing 100 ml of BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 400 μg l^{-1} biotin, and 0.1 M potassium phosphate, pH 6.0). *P. pastoris* was grown overnight at 30°C at 200 rpm, and recovered by

centrifugation the following day when the absorbance was between 2 and 6 units. Pellets from five flasks were pooled and resuspended in 100 ml of BMMY medium (1% yeast extract, 2% peptone, 400 µg l^{-1} biotin, 1% methanol, and 0.1 M potassium phosphate, pH 6.0) in a 500 ml flask. Induction was carried out for three days with the addition of three ml methanol per flask per day. The supernatant was then collected and after setting the pH to 7.8 with NaOH 1M, it was filtered through 0.22 µm filter membrane (Durapore GV membrane filters, 0.22 µm, Millipore, Molsheim, France). A HisTrap HP column (16 mm i.d., 5 ml 25 mm, GE Healthcare, France), prepacked with Ni High Performance Sepharose, was connected to an Äkta purifier chromatography system (GE Healthcare, France) and equilibrated with the equilibration buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 10 mM imidazole) before purification according to the instructions of the manufacturer. Protein was eluted with 50 mM Tris-HCl pH 7.8, 150 mM NaCl, 250 mM imidazole. Elution was monitored by measuring the absorbance at 280 nm. The fractions corresponding to the eluted protein were pooled, and loaded onto an ultrafiltration column (Vivaspin 3 or 10 kDa MWCO, PES, Sartorius, Palaiseau, France) for concentration and buffer exchange at 4 °C. The proteins were stored at 4 °C in a 50 mM sodium acetate buffer, pH 5.2. The concentration of the pure proteins was determined by measuring the absorbance of the solution at 280 nm on a Nanodrop 2000 (Thermo Fisher Scientific, France) and calculated using the Beer's law and the extinction coefficient of the protein as determined by ProtParam (http://web.expasy.org/protparam/).

Production of antibodies, protein electrophoresis and western immunoblotting

A solution of 800 µg of purified recombinant LbGH5-CBM1 protein was used to elicit rabbit polyclonal antibodies according to the manufacturer's procedure (Eurogentec, Seraing, Belgium). Total proteins from free-living mycelium, 15 ectomycorrhizal roots and 15 non-mycorrhizal roots were extracted according to Pitarch *et al*. (2002). Protein analyses were carried out by using 4-20% Mini-PROTEAN TGX Precast Protein gels (Bio-Rad, France) using a Mini-PROTEAN electrophoresis cell system (Bio-Rad, France). The specificity of the antibodies were determined by western blot of total protein obtained from poplar lateral roots not in contact with *L. bicolor* S238N and from

mycorrhizal root tips using the Bio-Rad alkaline phosphatase immun-blot kit(Bio-Rad Laboratories) according to manufacturers instructions.

Enzymatic assays

The enzyme activity of LbGH5 and LbGH5-CBM1 was assayed using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) from the amount of reducing sugar ends released during incubation with polysaccharide substrates (Couturier *et al.*, 2011). LbGH5 and LbGH5-CBM1 were incubated with the following polysaccharides: low viscosity carboxymethylcellulose (CMC, sodium salt, C5678, Sigma-Aldrich, France), pectin (P9135, Sigma Aldrich, France), 1,4-β-D-mannan (P-MANCB, Megazyme, Brey, Ireland), low viscosity galactomannan (P-GALML, Megazyme, Brey, Ireland), debranched sugar beet arabinan (P-DBAR, Megazyme, Brey, Ireland), larch wood arabinogalactan (P-ARGAL, Megazyme, Brey, Ireland), beechwood xylan (Sigma Aldrich), wheat arabinoxylan (Megazyme, Brey, Ireland), xyloglucan (tamarind seed, P-XYGLN, Megazyme, Brey, Ireland), and Avicel PH-101 (11365, Sigma Aldrich, France). Briefly, 100 nM of enzyme was mixed with 0.5% (W/V) substrate in 50 mM sodium acetate buffer pH 5.2. The reactions mixtures were incubated at 50°C, 850 rpm for 18 hours in a thermomixer (Eppendorf, Montesson, France). The samples were centrifuged and 150 µl of 1% DNS (w/v) reagent was added to 150 µl of each supernatant. The samples were heated at 95°C for 10 minutes. The reaction mixtures were cooled at room temperature, and 80 µl were transferred to a microtiter plate. Production of reducing sugar ends was determined by measuring the absorbance at 540 nm, and a glucose standard curve (0 to 10 mM) was used to calculate the release of sugar ends. Thin layer chromatography (TLC) was carried out as described in Couturier *et al.* (2011) to confirm the substrate hydrolysis.

Qualitative Binding Assays

Proteins (80 µg) were mixed with the following insoluble polysaccharides: 1% Avicel (w/v), 1% shrimp chitin (w/v), or 1% mannan (w/v) in a final volume of 200 µl containing 50 mM sodium acetate buffer, pH 5.2. Tubes were incubated on ice for 1 h with gentle mixing before being centrifuged at 13,000 *g* for 5 min, and the supernatants (containing the unbound proteins) were carefully removed. The polysaccharide pellets

were washed by resuspending in buffer and centrifuged. This step was done twice. Only the supernatant corresponding to the second wash was analyzed on gel. The remaining pellet was finally resuspended in SDS-loading buffer without dye (with a volume equivalent to the unbound fraction) and boiled for 10 min to dissociate any bound protein. Twenty-five µl of unbound, wash, and bound fractions were analyzed by SDS-PAGE on a 12% acrylamide gel.

Analysis of the hydrolysis products of recombinant proteins using high-performance anion-exchange chromatography

The degradation patterns and hydrolysis products of recombinant proteins were characterized by high-performance anion-exchange chromatography (HPAEC). Briefly, 6.8 µM of LbGH5-CBM1 were incubated with 1.25% (w/v) cellulose and 0.9% (w/v) hemicellulose purified from grey poplar (Schädel *et al*., 2010), 1.05% (w/v) *L. bicolor* cell walls (Wawra *et al*., 2016), or 1% (w/v) CMC, phosphoric acid swollen cellulose (PASC), laminarin (L9634, Sigma-Aldrich, France), lichenan, curdlan, or barley β-glucan (Megazyme, Ireland) in 50 mM citrate phosphate buffer (pH 4.5) for 15 min at 50 °C. To determine the effect of the CBM1, 253 nM of either LbGH5-CBM1 or LbGH5 were incubated with 1% (w/v) PASC, as described above. The reactions were stopped by heating at 95 °C for 15 min, the reaction mixture was centrifuged at 14,000g and the supernatant was analyzed for its carbohydrate composition with a HPAEC system equipped with pulsed amperometric detection (PAD), a CarboPac PA1 column (4 × 250 mm; Dionex, Thermo Scientific, Waltham, MA, USA), and a Carbopac PA1 guard column (4 × 50 mm, Dionex). The column was maintained at 30 °C, and was pre-equilibrated with 130 mM NaOH for 8 min. Then 10 µl samples were injected and eluted at 1 ml min^{-1} with a linear gradient of sodium acetate from 0 to 195 mM in 25 min, followed by isocratic elution for 5 min. Enzymatic reaction products were identified and quantified based on glucose, cellobiose (Sigma-Aldrich, France), cellotriose, cellotetraose, cellopentaose and cellohexaose standards (Megazyme, Ireland). The temperature tolerance was determined using CMC as substrate, whereas the pH optimum was determined using PASC, in the following 50 mM buffers: citrate phosphate (pH 3.45, 4.5, 5.8, 6.7, 7.4); acetate (pH 4.2 and 4.9); sodium carbonate (pH 9.1, 10.0, 10.9).

Confocal microscopy and indirect immunofluorescent localization

Three-week-old ectomycorrhizal root tips from grey poplar (cv. 717-1-B4) were fixed for 24 h in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM K_2HPO_4, pH 7.4). The root segments were embedded in agarose 5% (w/v) and cut into 25 µm longitudinal or 25 to 30 µm radial sections with a Leica VT1200S Leica vibratome (Leica Microsystems, Nanterre, France). Radial sections were sampled from three different regions of the mycorrhizal rootlets (100, 200 and 600 µm from the root apex) to assess LbGH5-CBM1 protein accumulation at different stages of ectomycorrhiza formation. To characterize the different developmental stages of ectomycorrhizae, sections were retrieved with a brush and carefully transferred onto watch glasses and then were stained according to Felten *et al.* (2009). For the indirect immunofluorescent (IIF) localization of the LbGH5-CBM1 protein was performed by confocal microscopy as described in Martin *et al.* (2008) (see also Supplemental Material online).

Results

L. bicolor *contains a single GH5 protein with a CBM1 domain*

Subfamily 5 of the glycoside hydrolases (GH5) comprises a group of 1,4-ß-D-glucan hydrolases (http://www.cazy.org/) that cleave different glucan polymers and are widespread in fungi. The haploid genome of *L. bicolor* S238N-H82 encodes a single polypeptide belonging to the GH5_5 subfamily (JGI ID# 319772). This GH5_5 protein is appended to the sole cellulose-binding module 1 (CBM1) domain found in the *L. bicolor* gene repertoire and has thus been named LbGH5-CBM1. The *LbGH5-CBM1* gene is 2,475 nt long and harbors 16 exons. The predicted transcript full length is 1,581 bp and the deduced polypeptide sequence contains 526 amino-acids with a corresponding MW of 54.5 kDa and theoritical pI equivalent to 4.79. The polypeptide contains a predicted signal peptide (position 1 to 24), a CBM1 domain (positions 25 to 57) and a GH5_5 catalytical module (positions 217 to 526) (Fig. 1a, Fig S1). LbGH5-CBM1 is thus a modular enzyme with a N-terminal CBM1 module appended to the catalytic module by an unusual long linker sequence (160 amino-acids) rich in serine and threonine residues, suggesting extensive *O*-glycosylation. Interestingly, the GH5_5

ortholog from *Laccaria amethystina* (GenBank ID KIJ95778), whose sequence is strictly identical to the *L. bicolor* GH5_5 in the CBM1 and catalytic module region only differs in the linker region that is 56 residues long (Fig. S2). The linker region in modular GH5_5 endoglucanases is generally much shorter than in *L. bicolor* GH5_5 (Toda *et al*., 2005).

The LbGH5-CBM1 sequence of *L. bicolor* S238N-H82 is highly similar (92 to 94 % identity) to the orthologous polypeptides from other *L. bicolor* strains from Europe and North America (data not shown). Sequence identity with the LaGH5-CBM1 from the sister species *L. amethystina* is 91% (Fig. S2), indicating that this endoglucanase is highly conserved within the *Laccaria* genus. A BLASTP query of *Lb*GH5-CBM1 against the NCBI nonredundant database followed by a phylogenetic analysis of fungal GH5_5 demonstrated that the *L. bicolor* protein grouped with the GH5_5 from *L. amethystina*, and the saprotrophic basidiomycetes *Galerina marginata* (74%) and *Volvariella volvacea* (72%) (Fig. 1b).

LbGH5-CBM1 expression is upregulated upon ectomycorrhiza development

The constitutive level of *LbGH5-CBM1* transcripts are barely detected by qPCR in the free living mycelium of *L. bicolor* S238N grown on Pachlewski agar medium (containing 0.55 to 5.5 mM glucose). Its expression is 18- to 26-fold lower than transcripts encoding a GH16 endo-1,3-β-glucanase (Fig. 2), an enzyme known to be involved in fungal cell wall synthesis remodelling. The presence of 0.25% (w/v) insoluble cellulose or a cellulose (cellophane) membrane (Fig 2) in the growth medium was not able to induce the *LbGH5-CBM1* transcription. In contrast, RNA sequencing from *Populus-L. bicolor* ectomycorrhizae has shown that the expression of *LbGH5-CBM1* is substantially upregulated (5- to 20-fold) during symbiosis development (Veneault-Fourrey *et al*., 2014; unpublished results). We confirmed the mycorrhiza-induced expression of *LbGH5-CBM1* by qPCR (Fig. 3a). *LbGH5-CBM1* was induced 25-fold in ectomycorrhizae after two weeks post contact, while it remained at a very low, constitutive level in free-living mycelium (Fig. 3a) and extraradical mycelium connected to ectomycorrhizal roots (data not shown).

LbGH5-CBM1 expression is required for ectomycorrhiza development

To assess the biological role of LbGH5-CBM1, knock-down RNAi lines were generated. We then checked the lower expression of *LbGH5-CBM1* in these knock-down RNAi lines and its impact on the morphology and anatomy of ectomycorrhizal roots. The *LbGH5-CBM1* transcription in the free-living mycelium of RNAi mutant lines (*A4, G4, H4*) was barely detectable and similar to the levels measured in wild-type S238N or mock (empty vector) control strains. *LbGH5-CBM1* transcripts in knock-down mycelium colonizing the poplar roots was also transcibed at a much lower level in 3-week-old ectomycorrhizas, indicating an efficient target gene RNA silencing, while in the mock control strain, *LbGH5-CBM1* expression induction was comparable with the wild-type fungus (Fig. 3a). The transcription of *LbGH5-CBM1* of RNAi mutant lines decreased by 83 to 96% by comparison to the empty vector controls.

This lower *LbGH5-CBM1* transcript abundance affects mycorrhizal formation as evidenced by phenotypic analysis (Fig. 3b). A major phenotypic feature of ectomycorrhizae established by *LbGH5-CBM1* RNAi mutant lines is the ability of the root apices to resume their apical growth and break the fungal mantle (Fig. 3d). This rare phenotype in the wild-type ectomycorrhizae increased by 35% in ectomycorrhizae established by *LbGH5-CBM1* RNAi mutant lines (Fig. 3b). In addition, laser-scanning confocal microscopy images showed that the colonizing hyphae of *LbGH5-CBM1* RNAi mutant lines differentiated a thinner uniseriate Hartig net between cortical cells, while the wild-type strain produced much larger Hartig net hyphae (Fig. 3e-f). The surface of the Hartig net hyphae is 16.12 $\mu m^2 \pm 0.38$ μm^2 per root section for the RNAi mutant lines, whereas this area is 37.78 $\mu m^2 \pm 2.86$ μm^2 for the wild-type mycorrhizas ($P<0.01$, Student *t* test, n=3-10).

A molecular marker of the Hartig net formation in *L. bicolor-Populus* ectomycorrhizae is the striking upregulation of the gene coding for the mycorrhiza-induced small secreted protein of 7 kDa, MiSSP7 (Plett *et al*. 2011). *L. bicolor MiSSP7* RNAi knock-down mutants are able to form a mantle sheath, but no intraradicular hyphal network (i.e., Hartig net) (Plett *et al*. 2011). The *MiSSP7* transcript abundance was 10-fold lower in ectomycorrhizae established by *LbGH5-CBM1* RNAi mutant lines (Fig. 3a) (relative to the wild-type strain), suggesting that the Hartig net formation is partly defective in *LbGH5-CBM1* RNAi mutant lines.

Cloning, expression and purification of LbGH-CBM1 and derived constructs in P. pastoris

The *L. bicolor* GH5_5 with and without its CBM1 domain was successfully expressed in *P. pastoris*, and purified by affinity chromatography. High production level and purification yields were obtained for LbGH5-CBM1 and LbGH5 enabling further biochemical characterization (Table 1). SDS-PAGE showed that the recombinant LbGH5-CBM1 and LbGH5 migrated as single bands with an estimated molecular mass of 130 and 35 kDa, respectively (Fig. 4a). Western blotting using anti-His tag antibody confirmed the high purity of the proteins produced in *P. pastoris* (Fig. 4b). The apparent molecular mass of LbGH5-CBM1 is higher (~130 kDa) than predicted based on the amino acid composition (i.e., 52.12 kDa). This is likely explained by the unusually long polylinker region causing conformational rearrangements and glycosylation of the recombinant protein in *P. pastoris*. However, incubation of the recombinant LbGH5-CBM1 with N- and O-deglycosylation enzymes did not change the apparent molecular mass of LbGH5-CBM1 (Fig. S4), suggesting that the electrophoretic mobility of the protein is affected by conformational rearrangements. It should be keep in mind that linker are mainly O-glycosylated and those glycosylation are hard to remove.

The purified recombinant LbGH5 protein (devoid of signal peptide and polylinker) was used to produce polyclonal antibodies in rabbit. Recombinant LbGH5-CBM1 and LbGH5 proteins were detected using these anti-LbGH5 antibodies by western immunoblotting (Fig. 4c). In soluble protein extracts from free-living mycelium and ectomycorrhizae, a single band at 53 kDa corresponding to LbGH5-CBM1was detected. The immune serum did not cross-react with soluble proteins extracted from poplar roots (Fig. 4d).

Lb*GH5-CBM1 has a functional secretion signal in the yeast system*

The yeast secretion trap (YST) assay showed that the predicted signal peptide of *Lb*GH5-CBM1 is able to rescue the growth of the *suc2* yeast mutant on sucrose medium (Fig. 5), indicating that the signal peptide of *Lb*GH5-CBM1 is capable of directing the protein secretion in a heterologous system.

Lb*GH5-CBM1 is an endoglucanase acting on poplar cellulose*

The substrate specificity of LbGH5-CBM1 and LbGH5 was determined from the amount of reducing sugars released during incubation with a series of polysaccharides, including low viscosity CMC, pectin, 1,4-β-D-mannan, low viscosity galactomannan, debranched sugar beet arabinan, larch wood arabinogalactan, water soluble and unsoluble wheat xylan, xyloglucan, and Avicel (microcrystalline cellulose). Purified LbGH5 and LbGH5-CBM1 appeared to act in a β-1,4 endoglucanase fashion, as indicated by the increase of reduced sugar ends from CMC, mannann and galactomannan (Fig. 6a). No activity on crystalline cellulose (Avicel), xyloglucan, xylan, arabinoxylan, arabinan or pectin was detected. Substrate hydrolysis was confirmed by TLC (Fig. S4).

The hydrolysis products of CMC, phosphoric acid swollen cellulose (PASC) and CMC yielded by the LbGH5-CBM1 and LbGH5 activity were analyzed by HPAEC-PAD. The purified LbGH5 and LbGH5-CBM1 mainly released decreasing amounts of cellobiose, cellotriose and cellotetraose, with low amounts of cellopentaose and glucose, as expected from a β-1,4 endoglucanase (Fig. 6b-d). The presence of the CBM1 domain increased the enzyme activity (Fig. 6a-d), but only a weak binding of CBM1 to cellulose was achieved with the purified CBM1 domain alone (data not shown). The optimum pH of the recombinant proteins was assayed in the presence of PASC as substrate.

The endoglucanase activity and patterns of hydrolysis products were also analyzed on cellulose and hemicellulose purified from roots of *P. trichocarpa*, and on *L. bicolor* cell wall polysaccharides. The HPAEC-PAD profile showed that LbGH5-CBM1 efficiently hydrolyzed poplar cellulose, releasing cellobiose and cellotriose as end products (Fig. 6e). In contrast, no activity was detected on poplar hemicellulose (data not shown) and *L. bicolor* cell walls (data not shown) The optimal pH of LbGH5-CBM1 and LbGH5 is 4.9 and 4.5, respectively (Fig. 6f).

Incubation of LbGH5-CBM1 in the presence of barley β-glucan (mixed linkage glucan with linear β-1,3 and β-1,4-bonds) released short β-1,4 linked cello-oligosaccharides and other unidentified oligosaccharides, presumably with mixed β-1,3/1,4 linkages (data not shown). No activity against pectin, xylan, arabinan,

arabinogalactan, xyloglucan (β-1,4-linked glucose with 1,6-linked xylose side chains), laminarin (β-1,3-linked glucose with β-1,6-linked intermittent or branching glucose residues), nor on pachyman, α/β-1,3-linked glucose polysaccharide was detected (data not shown).

LbGH5-CBM1 is a secreted endoglucanase accumulating at the periphery of hyphae

Polyclonal antibodies raised against the purified recombinant polypeptide were used for the localization of LbGH5-CBM1 in *L. bicolor* hyphae colonizing *P. tremula x P. alba* roots, i.e. 3-week-old ectomycorrhizas. Firstly, confocal microscopy using plant and fungal tissue staining was performed to characterize the different developmental stages of these mycorrhizal roots (Fig. 7). *L. bicolor* differentiates a thin mantle in the region behind the root cap (0 to 200 µm) (Fig. 7c,g), whereas a multilayer, thick mantle is formed in the mycorrhizal infection zone (200 to 400 µm) (Fig. 7b,f). Intense root colonization is also observed at the emergence of the lateral root (Fig. 7a,e).

IIF of ectomycorrhizal sections (200-400 µm) using anti- LbGH5 antibodies led to an intense labelling of hyphae constituting the mantle and penetrating the root to differentiate the Hartig net (Fig. 8b,c,f). The labelling was detected at the periphery of most of the hyphae (Fig. 8b) and oftenly coincided (Fig. 8c) with cell wall chitin labeling by the wheat germ agglutinin (WGA) (Fig. 8a), suggesting a cell wall and/or apoplastic localization. The labelling, i.e. accumulation of the *Lb*GH5-CBM1, is similar in the different regions of the mycorrhizal roots (i.e., root cap zone, mature mycorrhizal zone), (data not shown). In the control sections, where the preimmune serum was used instead of the anti-LbGH5 immune serum (Fig. 8e), no Alexa 488 IIF signal for *Lb*GH5-CBM1 was observed. In the presence of increasing concentration of the recombinant *Lb*GH5-CBM1 (competitive assay), the specific binding of antibodies to *Lb*GH5-CBM1 was precluded in the presence of 28 µg of recombinant *Lb*GH5-CBM1 and no signal was detected (Fig. 8d), confirming the high specificity of the immune serum.

Discussion

The increased expression of the *LbGH5-CBM1* gene encoding for a GH5 endoglucanase during symbiosis establishment in both *L. bicolor* (Martin *et al*., 2008; Veneault-

Fourrey *et al*., 2014) and *T. melanosporum* (Martin *et al*., 2010) suggests that the encoded enzyme may play a role in symbiosis development, i.e. fungal ingress in roots. The major objective of this study was to test this hypothesis by investigating the possible role of the mycorrhiza-induced LbGH5-CBM1 in root colonization by the ectomycorrhizal symbiont *L. bicolor* S238N. We have thus heterologously expressed, produced and characterized in terms of activity the LbGH5-CBM1 from *L. bicolor*, confirmed its role in mycorrhiza formation by RNAi knock-down of its gene expression, and determined its cellular and tissular localization.

The predicted product of the *LbGH5-CBM1* gene from *L. bicolor* shows conserved residues characteristic of the GH5_5 family (Fig. 1), thus suggesting that LbGH5-CBM1 can be classified as an endo-1,4-ß-glucanase pertaining to the GH5_5 family. The phylogenetic analysis of LbGH5-CBM1 show that this endo-1,4-ß-glucanase is closely related to other endo-1,4-ß-glucanases from saprotrophic fungi (Fig. 1). This might reflect similar substrate specificities of these fungal enzymes, namely cell walls of plant cells, or a similar evolutionary origin. YST assay confirmed that the predicted signal peptide of LbGH5-CBM1 is able to drive the secretion of the protein extracellularly. The recombinant LbGH5-CBM1 displays an endoglucanase activity towards CMC, PASC, (galacto)mannan and purified poplar cellulose. The enzyme was not active on poplar hemicellulose, crystalline cellulose (Avicel), xyloglucans, arabinans, and *L. bicolor* cell walls. Concerning the final hydrolysis products, LbGH5-CBM1 releases cellobiose, cellotriose and cellotetraose, with low amounts of cellopentaose and glucose, from CMC, PASC and poplar cellulose, as expected from a β-1,4 endoglucanase. LbGH5-CBM1 showed an optimal activity at pH 4 to 5, values similar to those of the plant apoplastic space. The CBM1 domain increases the optimal activity and thermostability of the GH5 enzyme has already observed for other fungal endoglucanases (Couturier *et al*., 2011). Transcription of the *LbGH5-CBM1* gene in the free-living mycelium is not upregulated by the addition of cellulose in the growth medium suggesting that its endoglucanase activity is not involved in the metabolic utilization of cellulose.

IIF confocal microscopy using anti-LbGH5 antibodies shows that the symbiosis-induced secreted *Lb*GH5-CBM1 endoglucanase is localized at the periphery of *L. bicolor* walls of hyphae colonizing the roots (Fig. 8). Its co-localization together

with, or adjacent to chitin, supports its extracellular/cell wall location. The accumulation of the endocellulase appears to be very high in the hyphae of the Hartig net (Fig. 8f). These hyphae are growing through a highly cross-linked network of polysaccharides, consisting of cellulose, hemicellulose and pectin, providing the cell shape tensile properties and hence the structural stability of the root epidermis. Modification of plant cell wall polymers would facilitate the fungal colonization of the host apoplastic space. The CBM1 domain of LbGH5-CBM1 presumably allows the secreted endoglucanase to access the cellulose component in the plant cell wall matrix during hyphal tip ingress, although CBM1 binding to cellulose in vitro was weak. Endoglucanase activity generates new chain ends by randomly cleaving internal bonds upon acting on cellulose chains. As *L. bicolor* is lacking GH6 and GH7 cellobiohydrolases, the plant cell walls are however not fully degraded, but by cleaving β-1,4 linkages between glucopyranose units, LbGH5-CBM1 likely alters the inherent strength of the host cell wall contributing to root penetration and Hartig net differentiation.

Intriguingly, the enzyme is not only found in the hyphae growing between root cells, i.e. in contact with its potential plant substrate, but also accumulates in the cell walls of hyphæ forming the mantle sheath. This suggests that signal(s) released by root cells induce the LbGH5-CBM1 gene expression and protein accumulation in a systemic way along the hyphal network. This pattern has been observed for several symbiosis-upregulated transcripts of *L. bicolor* colonizing poplar or Douglas fir rootlets (Kohler & Martin, unpublished results).

The *LbGH5-CBM1* gene is present in the *L. bicolor* genome as one copy. This allowed a functional analysis of the possible role of the endoglucanase in colonization of poplar roots by *L. bicolor* S238N. Knock-down of the *LbGH5-CBM1* gene expression by RNAi in *L. bicolor* indicates that the enzyme plays a substantial role in mycorrhiza development and more specifically in Hartig net formation. Mutant lines with a decreased level of *LbGH5-CBM1* transcripts established a lower number of mycorrhizal root tips. When mycorrhizas are established, they display a thinner Hartig net (Fig. 3f) and a lower expression of the Hartig net-molecular marker *MiSSP7* (Fig. 3a). In contrast to wild-type *L. bicolor* colonization which leads to the arrest of root meristem activity and apical growth (Vayssières *et al.*, 2015), the observation that a

substantial proportion of root tips colonized by knock-down *LbGH5-CBM1* RNAi mutants maintain their meristem activity leading to the breaking of the mantle sheath by root tips suggests that *L. bicolor* mutant lines have lost part of their control on root growth.

This is the first example for a possible role of a fungal cellulase in mutualistic interactions of ectomycorrhizal fungi with plants. It supports our contention that the few plant cell wall degrading enzymes remaining in the genomes of ectomycorrhizal fungi are not used for digesting the soil organic matter, but might play a role in the symbiosis establishment (Kohler *et al.*, 2015; Martin *et al*., 2016). Further analysis of mycorrhiza-induced CAZymes, such as the endopolygalacturonase GH28 will confirm the role of PCWDE in symbiosis development.

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Table 1. Biochemical characterization of recombinant *Lb*GH5-CBM1, *Lb*GH5 and *Lb*CBM1.

Protein	Apparent optimum pH	Apparent Molecular Mass (kDa)	
		Predicted	Experimental[a]
*Lb*GH5-CBM1	5.28	52.9	130.0[b]
*Lb*GH5	5.64	33.9	35.0
*Lb*CBM1	–	3.24	10.0

a. Values are estimated from SDS-PAGE

b. This higher value is likely the result of a high glycosylation of the linker

Legends of Figures

Figure 1. a, The modular stucture of the LbGH5-CBM1 endoglucanase from *L. bicolor* S238N-H82 showing the putative signal peptide, the cellulose-binding module 1 (CBM1) and the catalytic domain (GH5). **b**, A phylogenetic tree of fungal GH5_5 endoglucanases. The neighbor joining tree was generated by the NCBI plugin using the BLASTP output file of *L. bicolor Lb*GH5-CBM1 against the nonredundant NCBI database (max seq difference, 0.85; distance model, Grishin (protein)).

Figure 2. Transcription profiling of *LbGH5-CBM1* in *L. bicolor* free-living mycelium grown on increasing amount of glucose and cellulose. Transcripts of *LbGH5-CBM1* and the endo-1,3-β-glucanase *LbGH16* were measured by quantitative RT-PCR in RNA extracts. Data presented are the mean of three independent experiments ± SE. CM, the agar medium was covered with cellophane (cellulose) membrane.

Figure 3. *LbGH5-CBM1* expression is required for the formation of ectomycorrhizae. a, Relative expression of *LbGH5-CBM1* and the mycorrhiza-induced small secreted effector protein *MiSSP7* genes measured by quantitative RT-PCR in *LbGH5-CBM1* knock-down RNAi (*A4, G4, H4*), wild-type S238N and mock (lac ev7 and lac ev9, empty vector) control transformant strains colonizing poplar roots. FLM, wild-type S238N free living mycelium.
b, Mycorrhizal ability of knock-down RNAi *(A4, G4, H4*), wild-type S238N and empty vector (lac ev7 and lac ev9) control transformant strains. Note that knock-down RNAi established less mature ECM and more defective ECM than empty vector control and wild-type S238N strains. ***, P<0.001; **, δδ P< 0.01 and *, δP<0.05, versus *L. bicolor* S238N and empty vector, student's t test.
c and **d,** Images of mature and defective ectomycorrhizal root tips from *Populus tremula* x *alba* colonized by the wild-type S238N (**c**) or the knock-down *LbGH5-CBM1* RNAi *A4* (**d**) strains for three weeks; scale bar represents 80 μm. Laser-scanning confocal microscopy images of ectomycorrhizal root sections showing the mantle sheath and the intraradicular Hartig net established by the wild-type S238N (**e**) or the

knock-down *LbGH5-CBM1* RNAi *A4* (**f**) strains; transversal sections of 3-week-old ectomycorrhizae were stained for chitin with Alexa-WGA; scale bar represents 20 μm. Abbreviations, RT, root tip; PR, primary root; hn, Hartig net; ep, epidermal root cells; fm, fungal mantle; LR: lateral root; ECM: ectomycorrhizae. These images are representative of three different experiments.

Figure 4. SDS-PAGE and western blotting analysis of the recombinant LbGH5 and LbGH5-CBM1 proteins. a, SDS-PAGE of the recombinant proteins LbGH5-CBM1 and GH5 (without the CBM1 and linker); M, molecular mass protein markers (10 to 250 kDa). **b**, Western blot of the recombinant proteins LbGH5-CBM1 and GH5 detected by monoclonal antibodies against the polyhistidine (HIS) tag. **c**, Western blot of the recombinant proteins LbGH5-CBM1 and GH5 detected by a polyclonal anti-GH5 immune serum. **d**, Western blot of soluble proteins extracted from free-living mycelium of *L. bicolor* S238N (FLM), 3-week-old *P. tremula x alba-L. bicolor* ectomycorrhizae (ECM), and non-mycorrhizal roots of *P. tremula x alba* plantlets (poplar) probed by using anti-GH5 immune serum.

Figure 5. The signal peptide of LbGH5-CBM1 drives secretion of yeast SUC2 in the growth medium in the yeast secretion trap assay. a, Schematic representation of the Signal peptide-*SUC2* vector used for the Gateway construction. *SUC2* represents a yeast invertase gene lacking its own signal peptide (SP) and translation initiaton codon. **b**, Yeast strains transformed by the *LbGH5*_SP-*SUC2* construction or the *SUC2* construction without the LbGH5 signal peptide (LbGH5 W/O SP), respectively, were grown on a medium containing glucose (control medium) or sucrose (selection medium).

Figure 6. Endoglucanase activity of the recombinant LbGH5-CBM1 and LbGH5 proteins. (**a**) Hydrolytic activities of LbGH5-CBM1 and LbGH5 on different substrates. The assay mix composition was 0.5% (w/v) substrate and 100 nM enzyme in 50 mM sodium acetate buffer, pH 5.2. The samples were incubated at 50°C, 850 rpm during 18 hours in a thermomixer (Eppendorf, Montesson, France). The production of reducing ends was assayed by the dinitrosalicylic acid (DNS) reagent. HPAEC profiles

of reaction mixtures resulting from LbGH5-CBM1 (green), LbGH5 (red) and a GH16 positive control (black) activities, or with no enzyme (blue), using (**b**) CMC, (**c**) PASC, (**d**) Avicel, and (**e**) cellulose extracted from poplar roots (solid line), compared to the profile without enzyme (dashed line). In these assays, the recombinant LbGH5-CBM1 or LbGH5 (without CBM1) were incubated with the substrates at 50^{o}C for 15 min. Then, the enzymatic reactions were analyzed using HPAEC. Elution profiles were compared to cellobiose (1), cellotriose (2), cellotetraose (3) and cellopentaose (4) standards. **f**, Relative activity of LbGH5-CBM1 (green labels) and LbGH5 (black labels) at 50°C for 15 min in different buffers, (♦) 50 mM citrate buffer (pH 3.45-7.4), (■) 50 mM carbonate buffer (pH 9.1-10.9) and (▲) 50 mM acetate buffer pH (4.2-4.9). Values are based on the amount of sugars released from PASC and are relative to the maximum activity detected for each enzyme.

Figure 7. The different development stages of an ectomycorrhizal root of *Populus tremula x alba colonized by Laccaria bicolor*. All images were obtained by using immunofluorescence confocal laser microscopy. **a-d**, Longitudinal sections of a 3-week-old ectomycorrhizae were stained with DAPI (labelling plant nuclei in blue) and WGA- Alexa Fluor 633 (labeling fungal chitin in green). The whole longitudinal section is showed in the central panel (**d**), whereas different root regions (0-200 µm, 400-600 µm and >600 µm from the root tip) are highlighted on the left panel (**a-c**). Transversal sections taken at 200, 400 or 600 µm are showed on the right panel (**e-g**). Plant and fungal cell walls were stained with propidium iodide (red), whereas fungal chitin was stained with WGA-Alexa Fluor 488 (green). Abbreviations: AM, apical meristem; RC, root cap; PR, primary root; CC, cortical cell; hn, Hartig net; ep, epidermal cell; m, fungal mantle; scale bar, 20 µm.

Figure 8. Immunolocalization of LbGH5-CBM1 in *Populus tremula x alba–L. bicolor* ectomycorrhizae. All images were obtained by using indirect immunofluorescence confocal laser microscopy. Transversal sections of 3-week-old ectomycorrhizae stained for (**a**) chitin with WGA (red) or (**b**) LbGH5-CBM1 with anti-GH5 immune serum (green). (**c**) the merged image of (**a**) and (**b**) showing the co-localization of LbGH5-CBM1 and the fungal cell wall chitin. (**d**) The binding of the

anti-GH5 antibodies was blocked by incubating the sections in the presence of a solution of the recombinant LbGH5-CBM1 protein confirming the specificity of the immune serum. **(e**) the preimmune rabbit serum was used instead of the anti-LbGH5 immune serum. (**f**) The co-localization of LbGH5-CBM1 and the fungal cell wall chitin in a longitudinal section of ectomycorrhizae showing the accumulation of LbGH5-CBM1 in the labyrinthine hyphae of the Hartig net. Abbreviations, hn, Hartig net; ep, epidermal cell; fm, fungal mantle; scale bar, 20 μm.

Supplemental online material

Material & Methods

Confocal microscopy and indirect immunofluorescent localization

Sections were stained with wheat germ agglutinin (WGA) linked to either Alexa Fluor 488 or Alexa Fluor 633 conjugates (W11261 or W21404, ThermoFisher, France) which stains fungal cell walls in red or green, respectively. Plant cell walls were stained by propidium iodide, whereas plant and fungal nuclei were stained by DAPI. For the indirect immunofluorescent (IIF) localization of the LbGH5-CBM1 protein was performed by confocal microscopy as described in Martin *et al.* (2008). Sections were transferred onto watch glasses and incubated in 1% BSA for one hour. Then, BSA was removed and sections were incubated overnight with purified anti-LbGH5-CBM1protein rabbit antibody diluted 1:1,000 in PBS containing 0.5% (w/v) BSA at 4°C. The segments were then washed five times in PBS and incubated in the secondary antibody conjugate, a 1:500 dilution of goat anti-rabbit IgG-AlexaFluor 488 conjugate (A-11008, ThermoFisher, France) in PBS for 2 h. After five more washes in PBS, sections were mounted in 80% glycerol (Merck), 20% PBS, 5% w/v propyl gallate (Fluka) and viewed by a Zeiss LSM 800 microscope equipped with X10, X40, numerical aperture 1.4. The excitation and emission wavelengths for the Alexa Fluor 488 dye were 500 to 550 nm, respectively. Optical sections were collected at 0.1 to 0.7 mm intervals with Kalman averaging. As a control, sections were incubated with IgG purified from pre-immune serum diluted to the same concentration as anti-LbGH5-CBM1 IgG. In addition, we performed a competition epitope binding assay by incubating sections with both anti-LbGH5-CBM1 protein rabbit antibody diluted 1:1,000 and increasing concentration of recombinant LbGH5-CBM1 protein in PBS containing 0.5% (w/v) BSA at 4°C.

Table S1. Gene specific primers used in this study

Figure S1. The GH5-CBM1 of *Laccaria bicolor* S238N-H82. (a) the amino acid sequence; the predicted signal peptide is underlined, the linker is in black and the GH5

sequence is in green; (**b**) The GH5_5 PFAM domain; (**c**) The CBM1 PFAM domain; (**d**) the nucleotide sequences of the gene with introns (black) and exons (orange) and coding (CDS) sequence (underlined, predicted signal peptide; black, linker and green, GH5).

Figure S2. The GH5-CBM1 of *Laccaria amethystina*. (a) the amino acid sequence, with the predicted signal peptide underlined, the linker in black and the GH5 sequence in green; (b) Alignment of the LbGH5-CBM1 protein sequences from *L. bicolor* and *L. amethystina* showing the high divergence of the linker region.

Figure S3. SDS-PAGE and western blot of the products of the deglycosylation assay of the recombinant proteins LbGH5 and LbGH5-CBM1. Left panel, SDS-PAGE of LbGH5, LbGH5-CBM1 or fetuin (glycoprotein control) incubated with a deglycosylation mix containing peptide:N-glycosidase F (36kDa), O-glycosidase (147kDa), neuraminidase (43kDa), β-1,4 galactosidase (94kDa) and β-N-acetylglucosaminidase (71kDa) removing glycans from both O-linked and N-linked glycosylation sites. The apparent molecular weight (MW) of the control fetuin glycoprotein decreased from 50.0 kDa to 42.7kDa after treatment, while the MW of LbGH5 and LbGH5-CBM1. Right panel, western blot of the deglycosylation assay products using anti-LbGH5 antibodies.

Figure S4. Thin-layer chromatography of the enzyme products released by the LbGH5 and LbGH5-CBM1 recombinant proteins. Substrates (0.5%, w/v) were incubated with 100 nM enzyme in 50 mM reaction buffer (pH5.2) at 50°C (850 rpm) for 19h.