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Research

The ectomycorrhizal basidiomycete *Laccaria bicolor* releases a secreted β-1,4 endoglucanase that plays a key role in symbiosis development

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Summary

- In ectomycorrhiza, root ingress and colonization of the apoplast by colonizing hyphae is thought to rely mainly 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 yet been identified. 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, LbGH5-CBM1.
- Here, we characterize *LbGH5-CBM1* gene expression and the biochemical properties of its protein product. We also immunolocalized LbGH5-CBM1 by immunofluorescence confocal microscopy in poplar ectomycorrhiza.
- We show that *LbGH5-CBM1* expression is substantially induced in ectomycorrhiza, and RNAi mutants with a decreased *LbGH5-CBM1* expression have a lower ability to form ectomycorrhiza, suggesting a key role in symbiosis. Recombinant LbGH5-CBM1 displays its highest activity towards cellulose and galactomannans, but no activity toward *L. bicolor* cell walls. *In situ* localization of LbGH5-CBM1 in ectomycorrhiza reveals that the endoglucanase 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 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 into a fully developed pseudoparenchymatous sheath enclosing the root apex (Massicotte *et al.*, 1989). Hyphae then penetrate the root intercellularly after a loose hyphal weft has been formed between two rhizodermal cells to differentiate an intraradicular hyphal network, the so-called Hartig net (Balestrini & Kottke, 2016). 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, 2016). This channel-like hyphal structure is the most prominent feature of ectomycorrhiza. A common apoplast, the so-called symbiotic interface, resulting from the fusion of fungal and plant cell wall polysaccharides and proteins differentiates between plant cells and fungal hyphae favoring 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, 2016). Although Hartig net development does not lead to substantial qualitative change in cell wall composition, subtle alterations, such as localized loosening and swelling and redistribution of un-esterified pectins, accompany 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).

Root ingress and colonization of the host apoplast, for example, the middle lamella, by the colonizing hyphae is thought to rely mainly 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 (Gea *et al.*, 1994) to ease 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 (PCWDEs), such as symbiosis-upregulated endoglucanases and polygalacturonases (Peterson & Massicotte, 2004; Martin *et al.*, 2008; Veneault-Fourrey *et al.*, 2014). No experimental data have yet been produced to support either one of the above-mentioned hypotheses.

Cellulose is the major constituent of plant cell walls and its complete breakdown by fungal enzymes probably involves 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 kept in mind that the genome of the ectomycorrhizal *Laccaria bicolor* (Martin *et al.*, 2008), *Tuber melanosporum* (Martin *et al.*, 2010) and other ectomycorrhizal basidiomycetes sequenced so far (Kohler *et al.*, 2015) have no cellobiohydrolases from the GH6 or GH7 family, 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 GH5 family 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), suggesting that this endoglucanase may play a role in symbiosis development.

In this study, we confirmed the role of the *LbGH5-CBM1* gene during symbiosis development by confirming its increased expression in ectomycorrhiza and showing that RNAi mutants of *L. bicolor* have a reduced ability to form ectomycorrhiza. We then purified the recombinant LbGH5-CBM1 and showed that the enzyme is a secreted endoglucanase able to cleave cellulose from poplar roots and (galacto)mannans. 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 that the symbiosis-induced LbGH5-CBM1 endoglucanase plays a key role during symbiosis formation.

Materials 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 d (Felten *et al.*, 2009) before mycelia were harvested for mycorrhizal inoculation or further analyses of enzymatic activities and gene expression.

To assess the effect of increasing concentration of glucose and cellulose on expression of *LbGH5-CBM1*, 10-d-old mycelium was cultured in 100 ml modified low-glucose Pachlewski medium containing glucose (0.1, 0.05 or 0.01%, w/v) or 0.01% (w/v) glucose with 0.25% (w/v) α-cellulose (C8002, Sigma) in 250 ml E-flasks at 20°C in the dark on a shaker (200 rpm) for 7 d, 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).

Hybrid grey poplar (*Populus tremula* × *Populus alba*, INRA clone INRA 717-1-B4) was used for *in vitro* mycorrhizal inoculation. Two-centimeter-long poplar cuttings were rooted on solid Murashige and Skoog (MS) medium (Felten *et al.*, 2009) for 3 wk, and fungal mycelia 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 d. The membrane with *L. bicolor* mycelium and poplar seedlings with one or two main roots were transfered onto the surface of low-glucose Pachlewski agar medium (0.1% glucose) containing 0.1% MES (M8250, Sigma), and covered by another cellophane membrane. 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 3 wk after contact, together with nonmycorrhizal 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; 25–50 mycorrhizal root tips from these three plantlets were pooled into a single replicate.

Analysis of the *LbGH5-CBM1* gene

The haploid genome from *L. bicolor* S238N-H82 was sequenced and annotated as described by Martin *et al.* (2008) (http://genome.jgi.doe.gov/Lacbi2/Lacbi2.home.html, RefSeq: NZ_ABFE00000000.1). Carbohydrate-active enzymes were annotated as described by 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 probable subcellular 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 reverse transcriptase PCR (qRT-PCR)

Free-living mycelium (200 mg) and at least 25 mycorrhizal or nonmycorrhizal lateral rootlets sampled on three different root systems of *P. tremula* × *P. alba* clone INRA 717-1B4 colonized by *L. bicolor* (100 mg) were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the RNeasy Plant kit (Qiagen) 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). RNA quality was checked by using Experion HighSens capillary gels (BioRad). Synthesis of cDNA from 1 μg of total RNA was performed using the iScript kit (BioRad) 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) 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) 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) 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). Primer 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 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 for transformation of *L. bicolor* vegetative mycelium we used the pHg/pSILBAg vector system described by Kemppainen & Pardo (2010) using the cDNA fragments of *LbGH5-CBM1*. To generate mock transformant strains, *L. bicolor* S238N wild-type was Agro-transformed with pHg/pSILBAγ. This mock vector carried a full-length, *Sac*I-linearized, empty pSILBAγ 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 assays. 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 wk and in poplar ectomycorrhiza ($n = 3$, mean ± 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.

Yeast secretion trap assay

To construct a Gateway-compatible vector for yeast secretion (Plett *et al.*, 2011), 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) according to Lee *et al.* (2006). Secretion of the yeast invertase with LbGH5-CBM1 signal peptide was detected by yeast growth on 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 pPICZaαA 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. The nucleotide sequences of *LbGH5-CBM1*, *LbGH5* and *CBM1* (Fig. S1) were codon-optimized for expression in *P. pastoris* and synthesized by Genscript (Piscataway, 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 control of the *AOX1* promoter. The expression protocol is described by Couturier *et al.* (2011). Large scale (2.4 liter) production of each protein was performed in 500 ml nonbaffled 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 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 3 d with the addition of 3 ml methanol per flask per day. The supernatant was then collected and after setting the pH to 7.8 with 1 M NaOH, it was filtered through a 0.22 μm filter membrane (Durapore GV membrane filters, 0.22 μm, Millipore). A HisTrap HP

column (16 mm i.d., 5 ml 25 mm, GE Healthcare, Buc, France), prepacked with Ni High Performance Sepharose, was connected to an Äkta purifier chromatography system (GE Healthcare) 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 and 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 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) and calculated using 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 nonmycorrhizal roots were extracted according to Pitarch *et al.* (2002). Protein analyses were carried out by using 4–20% Mini-PROTEAN TGX Precast Protein gels in a Mini-PROTEAN electrophoresis cell system (both Bio-Rad). Specificity of the antibodies was 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 the manufacturer's 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), pectin (P9135, Sigma Aldrich), 1,4-β-D-mannans (P-MANCB; Megazyme, Brey, Ireland), low-viscosity galactomannans (P-GALML, Megazyme), debranched sugar beet arabinan (P-DBAR, Megazyme), larch wood arabinogalactan (P-ARGAL; Megazyme), beechwood xylan (Sigma Aldrich), wheat arabinoxylan (Megazyme), xyloglucan (tamarind seed, P-XYGLN; Megazyme) and Avicel PH-101 (11365; Sigma Aldrich). 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 h 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 min. The reaction mixtures were cooled at room temperature, and 80 µl was transferred to a microtiter plate. Production of reducing sugar ends was determined by measuring the absorbance at 540 nm, and comparing to a glucose standard curve (0–10 mM). Thin layer chromatography (TLC) was carried out as described by Couturier *et al.* (2011) to confirm substrate hydrolysis.

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 was 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), lichenan, curdlan or barley β-glucan (Megazyme) 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 was 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 14 000 ***g*** and the supernatant was analyzed for its carbohydrate composition with an HPAEC system equipped with pulsed amperometric detection (PAD), a CarboPac PA1 column (4 × 250 mm; Dionex, Thermo Scientific) 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), cellotriose, cellotetraose, cellopentaose and cellohexaose standards (Megazyme). 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 immunofluorescence localization

Three-week-old ectomycorrhizal root tips from grey poplar (cv INRA 717-1-B4) or free-living mycelium of *L. bicolor* S238N (wild-type and RNAi mutant lines) were fixed for 8 h in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (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 25–30 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. Sections were retrieved with a brush and carefully transferred onto watch glasses and then were stained according to Felten *et al.* (2009). The indirect immunofluorescent (IIF) localization of the LbGH5-CBM1 protein was performed by confocal microscopy as described by Martin *et al.* (2008) (see also Methods S1).

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-β-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 CBM1 domain found in the *L. bicolor* gene repertoire and has thus been named LbGH5-CBM1. The *LbGH5-CBM1* gene is 2475 nt long and harbors 16 exons (Methods S1). The predicted full-length transcript is 1581 bp and the deduced polypeptide sequence contains 526 amino acids with a corresponding molecular weight of 54.5 kDa and a pI of 4.79. The polypeptide contains a predicted signal peptide (position 1–24), a CBM1 domain (positions 25–57) and a GH5_5 catalytical module (positions 217–526; Fig. S1). LbGH5-CBM1 is thus a modular enzyme with an 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 accession number KIJ95778), whose sequence is strictly identical to the *L. bicolor* GH5_5 in the CBM1 and catalytic module region, differs only 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). 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 genus *Laccaria*.

LbGH5-CBM1 expression is upregulated in ectomycorrhiza

The constitutive level of *LbGH5-CBM1* transcripts was very low in the free-living mycelium of *L. bicolor* S238N grown on Pachlewski agar medium (containing 0.55–5.5 mM glucose). Expression was 18- to 26-fold lower than for transcripts encoding a GH16 endo-1,3-β-glucanase (Fig. 1a), an enzyme known to be involved in fungal cell wall remodelling. The presence of 0.25% (w/v) insoluble cellulose or a cellulose (cellophane) membrane (Fig. 1a) in the growth medium was not able to induce *LbGH5-CBM1* transcription. By contrast, RNA sequencing from *Populus–L. bicolor* ectomycorrhiza has shown that the expression of *LbGH5-CBM1* is substantially upregulated (five- to 20-fold) during symbiosis development (A. Kohler, unpublished results). We confirmed the mycorrhiza-induced expression of *LbGH5-CBM1* by qPCR (Fig. 1b). *LbGH5-CBM1* was induced 25-fold in ectomycorrhiza 2 wk after contact, while it remained at a very low, constitutive level in free-living mycelium (Fig. 1b). The *LbGH5-CBM1* gene transcripts was not detected in fruiting bodies (data not shown).

LbGH5-CBM1 expression is required for ectomycorrhiza development

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* was also expressed at low level in knock-down mycelium colonizing poplar roots, indicating an efficient target gene RNA silencing, while in the mock control strain, *LbGH5-CBM1* transcript levels were comparable to the wild-type fungus (Fig. 1b). The transcript levels of *LbGH5-CBM1* of RNAi mutant lines decreased by 83–96% compared to empty vector controls.

This lower *LbGH5-CBM1* transcript abundance affected mycorrhizal formation, as evidenced by phenotypic analysis (Fig. 1c). A major phenotypic feature of ectomycorrhiza established by *LbGH5-CBM1* RNAi mutant lines was the ability of the root apices to resume their apical growth and break the fungal mantle (Fig. 2a). This rare phenotype in the wild-type ectomycorrhiza (Fig. 2b) increased by 35% in ectomycorrhiza established by *LbGH5-CBM1* RNAi mutant lines (Fig. 1c). In addition, laser-scanning confocal microscopy images showed that the colonizing hyphae of *LbGH5-CBM1* RNAi mutant lines differentiated into a thinner uniseriate Hartig net between cortical cells (Fig. 2c), while the wild-type strain produced much larger Hartig net hyphae (Fig. 2d). The surface area of the Hartig net hyphae was $16.12 \pm 0.38\ \mu m^2$ per root section for the RNAi mutant lines, whereas this area was $37.78 \pm 2.86\ \mu m^2$ for the wild-type mycorrhizas ($P < 0.01$, Student's *t*-test, $n = 3–10$).

A molecular marker of Hartig net formation in *L. bicolor–Populus* ectomycorrhiza is the striking upregulation of the fungal 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). *MiSSP7* transcript abundance was 10-fold lower in ectomycorrhiza established by *LbGH5-CBM1* RNAi mutant lines (Fig. 1b) relative to the wild-type strain, suggesting that Hartig net formation is partly defective.

Cloning, expression and purification of LbGH5-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 concentrations and purification yields were obtained for LbGH5-CBM1 and LbGH5, enabling further

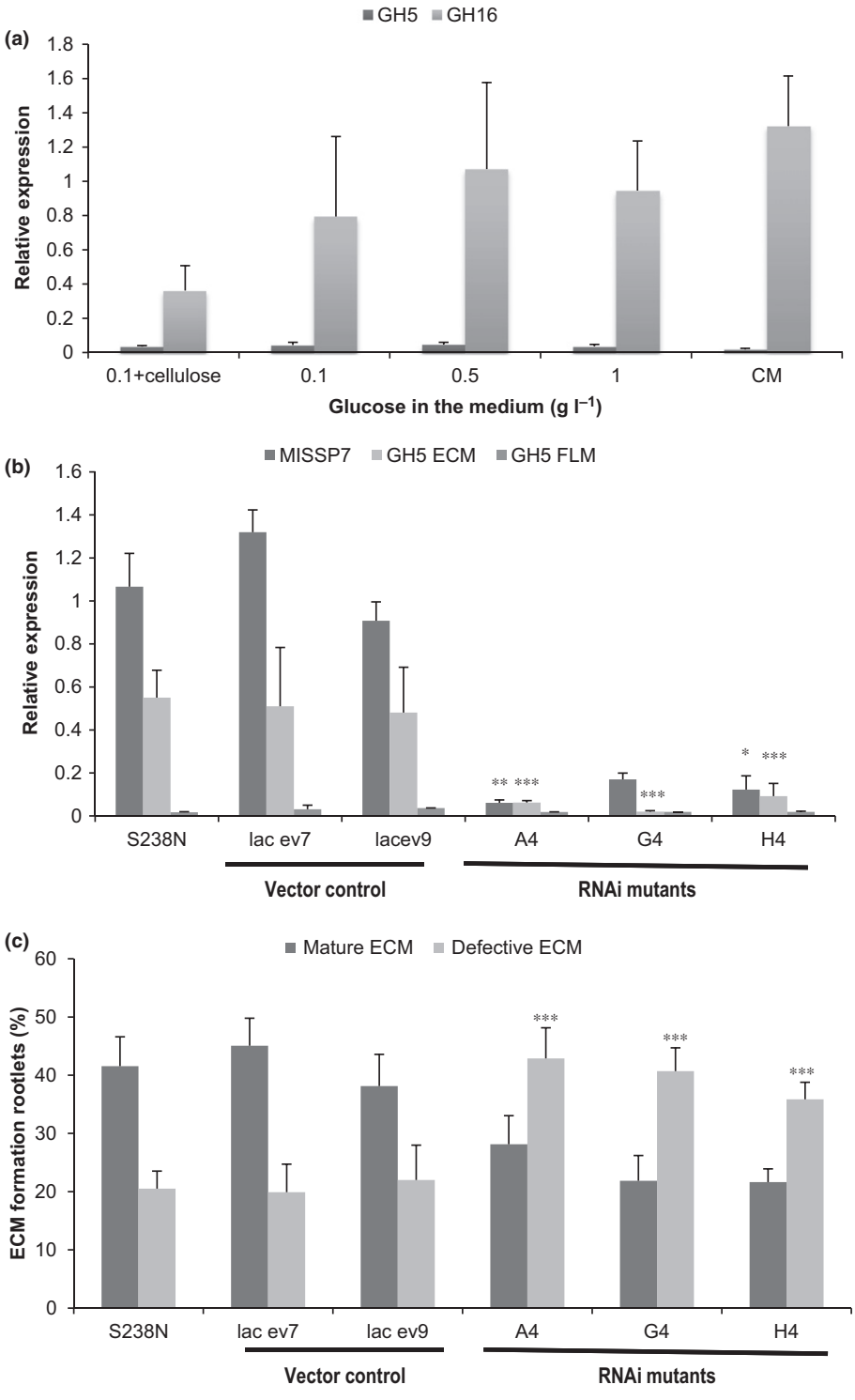

Fig. 1 Transcription profiling of *LbGH5-CBM1*. (a) Transcripts of *LbGH5-CBM1* and the endo-1,3-β-glucanase *LbGH16* in *Laccaria bicolor* free-living mycelium grown with increasing concentrations of glucose and cellulose were measured by qRT-PCR in RNA extracts. Data presented are the mean of three independent experiments ± SE. CM, the agar medium containing 1g l-1 glucose was covered with cellophane (cellulose) membrane. (b) Relative expression of *LbGH5-CBM1* and the mycorrhiza-induced small secreted effector protein *MiSSP7* genes measured by qRT-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. (c) 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 ectomycorrhiza (ECM) and more defective ECM than empty vector control and wild-type S238N strains. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, vs *L. bicolor* S238N and empty vector, Student's *t*-test.

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. 3a). Western blotting using anti-His tag antibody confirmed the high purity of the proteins produced in *P. pastoris* (Fig. 3b). The apparent molecular mass of LbGH5-CBM1 is higher (*c.* 130 kDa) than predicted based on the amino acid composition (i.e. 52.12 kDa). This is probably due to 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. S3), suggesting that the electrophoretic mobility of the protein is affected

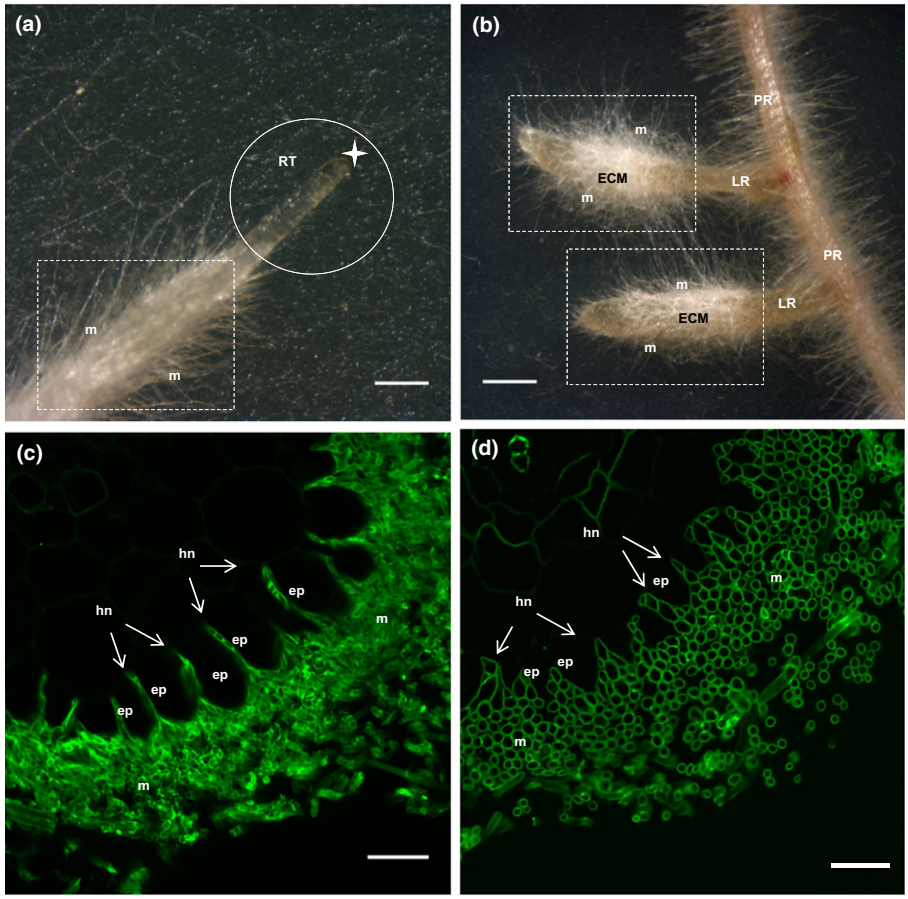

Fig. 2 *LbGH5-CBM1* expression is required for the formation of ectomycorrhiza. (a, b) Images of mature and defective ectomycorrhizal root tips from *Populus tremula x P. alba colonized by* (a) the knockdown *LbGH5-CBM1* RNAi *A4* strains or (b) the wild-type S238N for 3 wk. (c, d) Laser-scanning confocal microscopy images of ectomycorrhizal root sections showing the mantle sheath and the intraradicular Hartig net established by (c) the knockdown *LbGH5-CBM1* RNAi *A4* strains or (d) the wild-type S238N. Transverse sections of 3-wk-old ectomycorrhiza were stained for chitin with Alexa wheat germ agglutinin. RT, root tip; PR, primary root; hn, Hartig net; ep, epidermal root cells; m, fungal mantle; LR, lateral root; ECM, ectomycorrhiza. These images are representative of three different experiments. Bars: (a, b) 80 μm; (c, d) 20 μm.

Table 1 Biochemical characterization of recombinant LbGH5-CBM1, LbGH5 and LbCBM1

Protein	Apparent optimum pH	Apparent molecular mass (kDa)[a]	
		Predicted	Experimental[a]
LbGH5-CBM1	5.28	52.9	130.0[b]
LbGH5	5.64	33.9	35.0
LbCBM1	–	3.24	10.0

[a]Values are estimated from SDS-PAGE.
[b]This higher value is probably the result of a high glycosylation of the linker.

by conformational rearrangements. It should be kept in mind that linkers are mainly *O*-glycosylated and such glycosylation is 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. 3c). In soluble protein extracts from free-living mycelium and ectomycorrhiza, a single band at 53 kDa corresponding to LbGH5-CBM1 was detected. The immune serum did not cross-react with soluble proteins extracted from poplar roots (Fig. 3d).

*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. 4), 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 cellulose, mannans and galactomannans

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-mannans, low-viscosity galactomannans, 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, mannans and galactomannans (Fig. 5a). No activity on crystalline cellulose (Avicel), xyloglucan, xylan, arabinoxylan, arabinan or pectin was detected. Substrate hydrolysis was confirmed by TLC (Fig. S4).

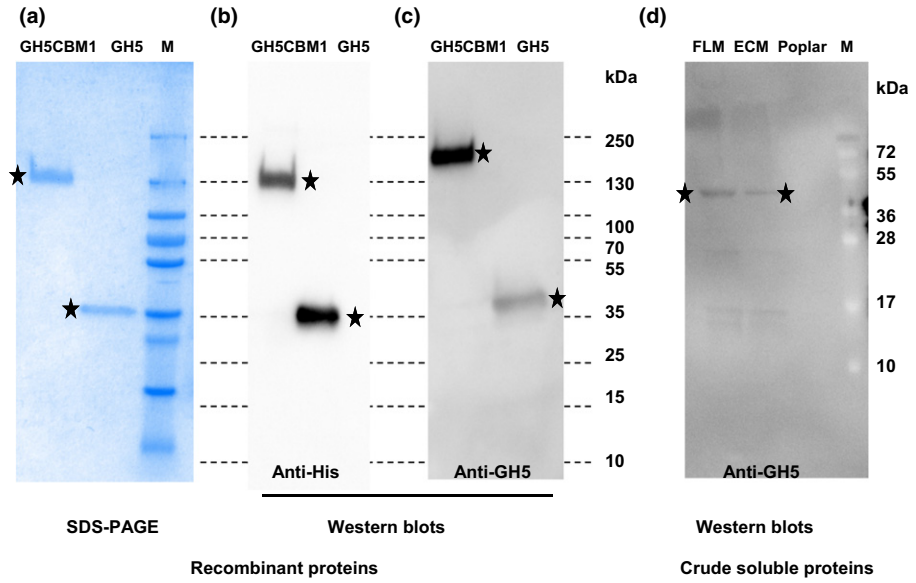

Fig. 3 SDS-PAGE and western blotting analysis of the recombinant LbGH5 and LbGH5-CBM1 proteins. (a) SDS-PAGE of the recombinant proteins LbGH5-CBM1 and GH5 (without the CBM1 and linker); M, molecular mass protein markers (10–250 kDa). (b) Western blot of the recombinant proteins LbGH5-CBM1 and GH5 detected 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 *Laccaria bicolor* S238N (FLM), 3-wk-old *Populus tremula* × *P. alba*–*L. bicolor* ectomycorrhiza (ECM), and nonmycorrhizal roots of *P. tremula* × *P. alba* plantlets (poplar) probed by using anti-GH5 immune serum.

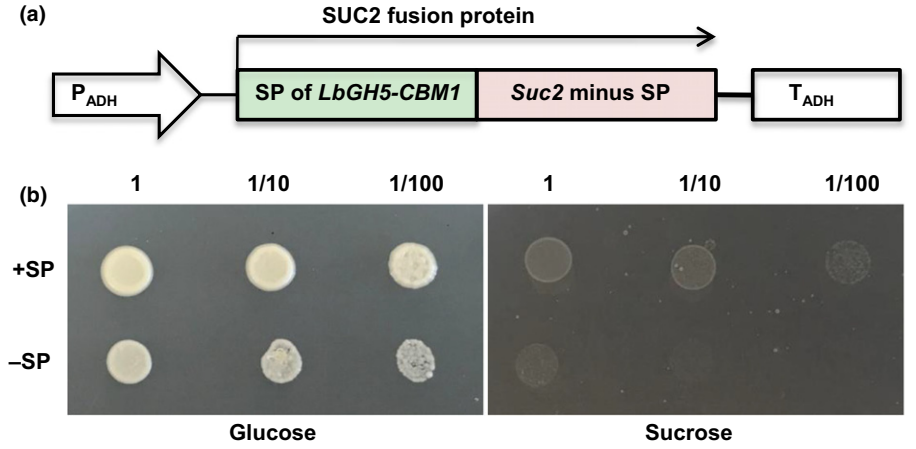

Fig. 4 The signal peptide of LbGH5-CBM1 drives secretion of yeast SUC2 in the growth medium in the yeast secretion trap assay. (a) Schematic representation of the signal peptide-*SUC2* vector used for the Gateway construction. *SUC2* represents a yeast invertase gene lacking its own signal peptide (SP) and translation initiation codon. (b) Yeast strains transformed by the *LbGH5*_SP-*SUC2* construct or the *SUC2* construct without the LbGH5 signal peptide (LbGH5 W/O SP), respectively, were grown on medium containing glucose (control medium) or sucrose (selection medium).

The hydrolysis products of 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. 5b–d). The presence of the CBM1 domain increased the enzyme activity (Fig. 5a–d), but only a weak binding of CBM1 to cellulose was achieved with the purified CBM1 domain alone (data not shown).

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. 5e). By contrast, no activity was detected on poplar hemicellulose (data not shown) and *L. bicolor* cell walls (data not shown). The optimum pH of the recombinant proteins was assayed in the presence of PASC as substrate. The optimal pH of LbGH5-CBM1 and LbGH5 is 4.9 and 4.5, respectively (Fig. 5f).

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 or 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* × *P. alba* roots, that is, 3-wk-old ectomycorrhiza. *L. bicolor* develops a thin mantle in the region behind the root cap (0–200 μm; Fig. S5a,b), whereas a multilayer, thick mantle is formed in the mycorrhizal infection zone (200–400 μm; Fig. S5c,d). Intense root colonization is also observed at the emergence of the lateral root (Fig. S5e,f). IIF of ectomycorrhizal sections (200–400 μm) using anti-LbGH5 antibodies led to an intense labeling of hyphae constituting the mantle and penetrating the root to differentiate into the Hartig net (Fig. 6). Labeling was mainly detected at the periphery of the hyphae (Fig. 6a) and it oftenly coincided (Fig. 6a) with the location of cell wall chitin labeled by wheat germ agglutinin (WGA; Fig. 6a), suggesting a cell wall and/or apoplastic localization. The labeling, that is, 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. 6b), no Alexa 488 IIF signal for *Lb*GH5-CBM1 was observed. In the presence of increasing concentrations 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. 6c), confirming high specificity of the immune serum. Although the *LbGH5-CBM1* transcripts are expressed at a low level in the free-living mycelium, the LbGH5-CBM1 protein was detected in the free-living mycelium, but it accumulated intracellularly (Fig. 6d).

Discussion

The increased expression of the *LbGH5-CBM1* gene coding for a GH5 endoglucanase during symbiosis establishment in *L. bicolor* (Martin *et al.*, 2008; Veneault-Fourrey *et al.*, 2014) suggests that the encoded enzyme may play a role in symbiosis development, that is, 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 produced the LbGH5-CBM1 from *L. bicolor* and characterized the enzyme in terms of activity, 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. S1), suggesting that LbGH5-CBM1 enzyme is an endo-1,4-β-glucanase. 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 displayed an endoglucanase activity towards CMC, PASC, (galacto)mannans and purified poplar cellulose. LbGH5-CBM1 activity released 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.

The activity against mannan polysaccharides (mannans and galactomannans) is expected for some members of the GH5_5 subfamily (Li & Walton, 2017). It can be explained by the fact that D-mannose and D-glucose are epimers and therefore cellulose and mannan are structurally similar. The enzyme was not active on poplar hemicellulose, crystalline cellulose (Avicel), xyloglucans, arabinans or *L. bicolor* cell walls. LbGH5-CBM1 showed an optimal activity at pH 4–5, 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. This inability to use cellulose as a carbon source is supported by the absence of *L. bicolor* growth on cellulose (Veneault-Fourrey *et al.*, 2014).

IIF confocal microscopy using anti-LbGH5 antibodies shows that the symbiosis-induced secreted LbGH5-CBM1 endoglucanase is localized at the periphery of walls of *L. bicolor* hyphae colonizing the roots (Fig. 6). Its co-localization together with, or adjacent to, chitin supports its extracellular/cell wall location. The accumulation of the endoglucanase appears to be high in the hyphae of the Hartig net (Fig. 6). 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 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 in 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 probably alters the inherent strength of the host cell wall contributing to root penetration and Hartig net differentiation. The endo-1,4-β-mannanase activity of LbGH5-CBM1 on mannanns and galactomananns suggests that the enzyme may also alter the composition of the mannoproteins accumulating at the surface of cell walls from ectomycorrhizal fungi and the host plant (Balestrini & Kottke, 2016).

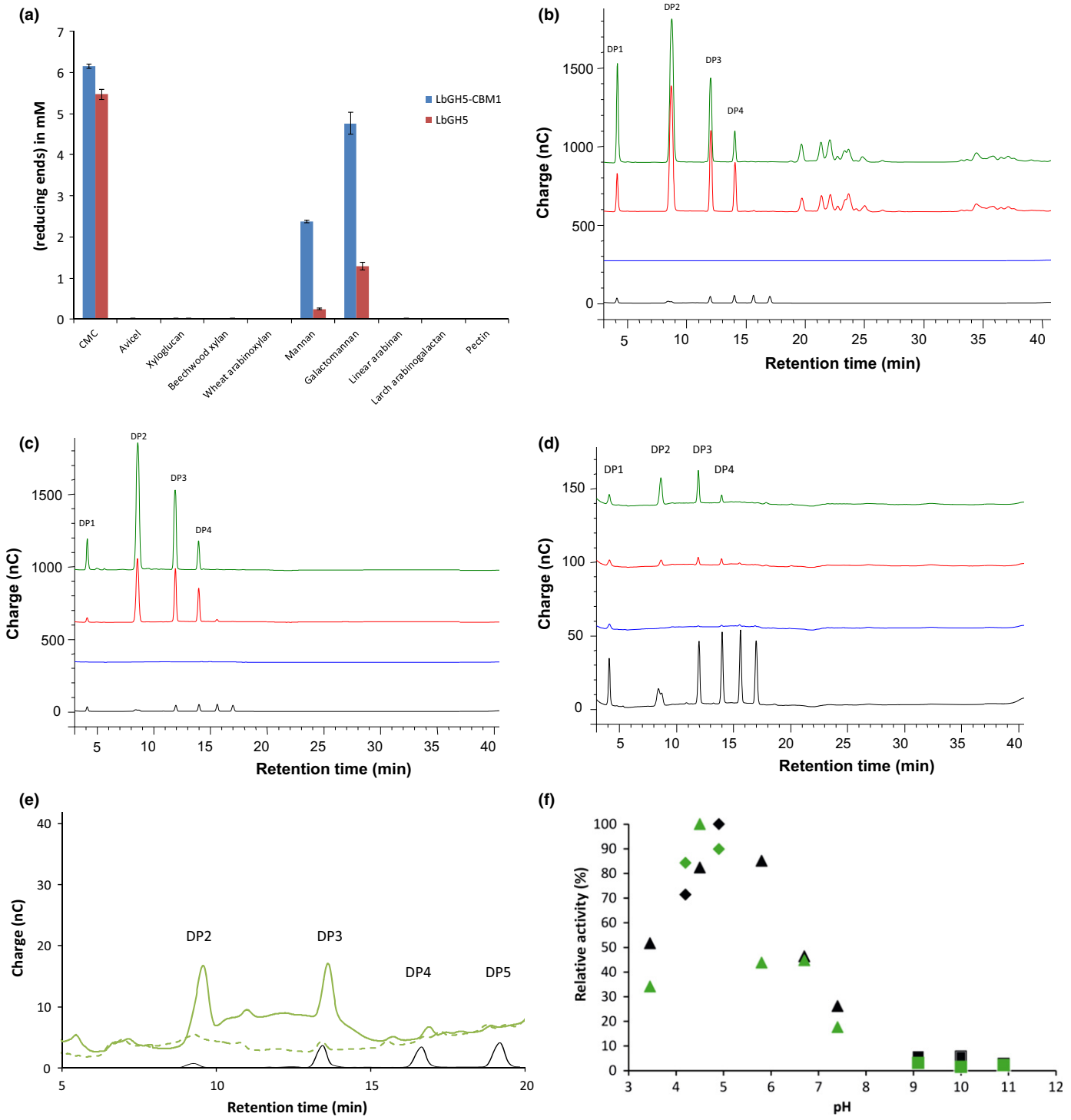

Fig. 5 Enzymatic activity of LbGH5-CBM1 and LbGH5 proteins. (a) Hydrolytic activities of LbGH5-CBM1 (blue bars) and LbGH5 (red bars) on different polysaccharides. The assay mix composition was 0.5% (w/v) substrate and 100 nM enzymes in 50 mM sodium acetate buffer, pH 5.2. The samples were incubated at 50°C, 850 rpm for 18 h. The production of reducing ends was assayed by the dinitrosalicylic acid (DNS) reagent. CMC, carboxymethylcellulose. Data presented are the mean of three independent experiments ± SE. (b–d) High-performance anion-exchange chromatography (HPAEC) profiles of LbGH5-CBM1 and LbGH5 reaction mixtures using (b) CMC, (c) phosphoric acid swollen cellulose (PASC) and (d) Avicel. (e) HPAEC profiles of LbGH5-CBM1 reaction mixtures using cellulose extracted from poplar roots (green solid lines) and *Laccaria bicolor* cell polysaccharides (green dashed lines). In these assays, LbGH5-CBM1 or LbGH5 were incubated with the substrates at 50°C for 15 min and the reaction mix was analyzed using HPAEC. Elution profiles (green, LbGH5-CBM1; red, LbGH5; blue, control) were compared to DP1–DP6 cello-oligosaccharides standards (black). Peaks are labeled as DP1 (glucose), DP2 (cellobiose), DP3 (cellotriose), DP4 (cellotatraose), DP5 (cellopentaose) and DP6 (cellohexaose). (f) Relative activity of LbGH5-CBM1 (green labels) and LbGH5 (black labels) at 50°C for 15 min in buffers with different pH, (diamonds) 50 mM citrate buffer (pH 3.45–7.4), (squares) 50 mM carbonate buffer (pH 9.1–10.9) and (triangles) 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 of the two variants of the enzyme.

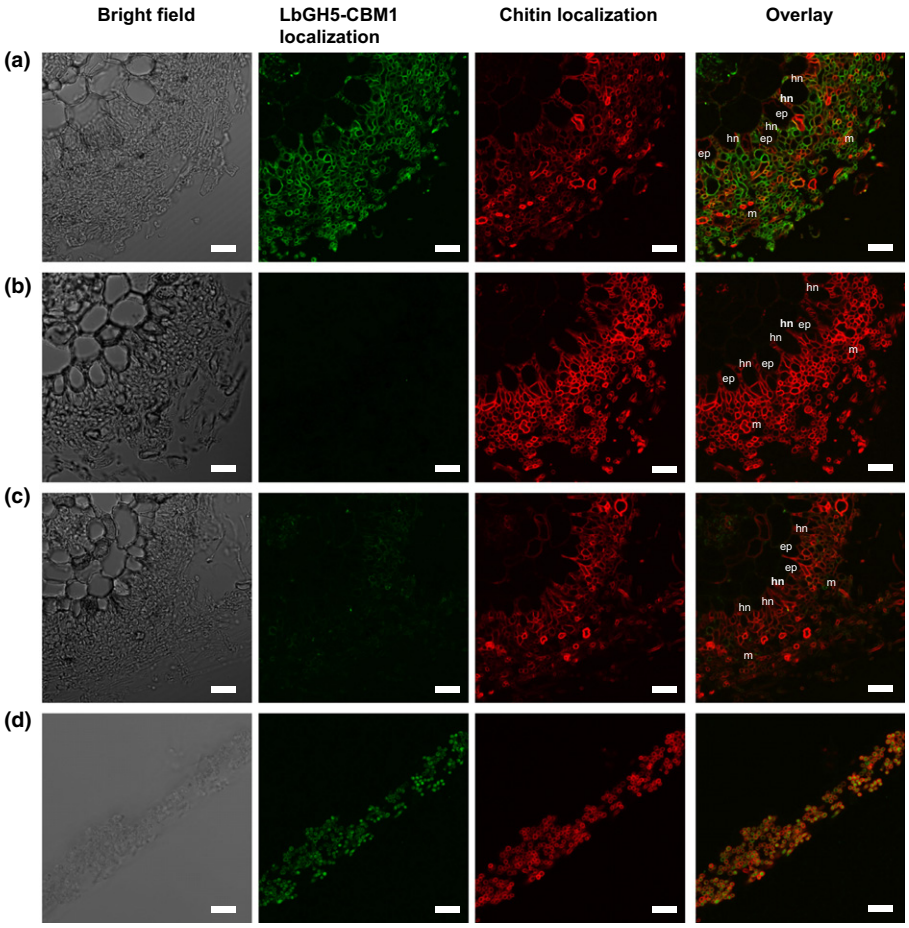

Fig. 6 Immunolocalization of LbGH5-CBM1 in *Populus tremula* × *P. alba* – *Laccaria bicolor* ectomycorrhiza. All images were obtained by using indirect immunofluorescence confocal laser microscopy, except the bright field images. (a) Transverse sections of 3-wk-old ectomycorrhiza stained for LbGH5-CBM1 with anti-GH5 immune serum (green) and for chitin with wheat germ agglutinin (WGA) (red). (b) The preimmune rabbit serum was used instead of the anti-LbGH5 immune serum. (c) 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. (d) Sections of *L. bicolor* free-living mycelium stained for LbGH5-CBM1 with anti-GH5 immune serum (green) and for chitin with WGA (red). Panels represent bright field (first row), LbGH5-CBM1 localization (second row), chitin localization (third row) and overlay of anti-LbGH5-CBM1 and chitin (fourth row). Bars, 20 μm.

Intriguingly, the enzyme is not only found in the hyphae growing between root cells, that is, in contact with its potential plant substrate, but also accumulates in the cell walls of hyphae 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 (A. Kohler & F. Martin, unpublished results). In the free-living mycelium, *LbGH5-CBM1* transcripts are present at a very low level (Fig. 1a,b), but the protein is detected by IIF confocal microscopy using anti-LbGH5 antibodies (Fig. 6d). It is only found intracellularly, suggesting symbiosis development leads to an increased expression of the *LbGH5-CBM1* gene, but also to localization of LbGH5-CBM1 to the cell wall and apoplastic space.

The *LbGH5-CBM1* gene is present as a single copy in *L. bicolor*. This allowed a functional analysis of the possible role of the endoglucanase in colonization of poplar roots by *L. bicolor* S238N. Knock-down of *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 reduced number of mycorrhizal root tips. When mycorrhizas are established, they display a thinner Hartig net (Fig. 2c) and a lower expression of the Hartig net molecular marker *MiSSP7* (Fig. 1b). 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 β-1,4 endoglucanase 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 soil organic matter, but might play a role in 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 PCWDEs in symbiosis development.

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Author contributions

F.M. and J.G-B. planned and designed the research. F.Z., G.E.A., A.L., C. Champion, M.H. M.K., C. Commun and A.D. performed experiments, F.Z., G.E.A., A.P., C.V-F., A.K., M-.N.R., B.H., J.G-B. and F.M. analyzed the data, and F.M., F.Z., G.E.A. and J.G-B. wrote the manuscript with the help of C.V-F., M-N.R. and B.H.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 The GH5-CBM1 of *Laccaria bicolor* S238N-H82.

Fig. S2 The GH5-CBM1 of *Laccaria amethystina*.

Fig. S3 SDS-PAGE and western blot of the products of the deglycosylation assay of the recombinant proteins LbGH5 and LbGH5-CBM1.

Fig. S4 Thin-layer chromatography of the enzyme products released by the LbGH5 and LbGH5-CBM1 recombinant proteins.

Fig. S5 The different development stages of an ectomycorrhizal root of *Populus tremula* × *P. alba* colonized by *Laccaria bicolor*.

Table S1 Gene-specific primers used in this study

Methods S1 Confocal microscopy and indirect immunofluorescence localization.

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