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(Article begins on next page)



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Understanding plant cell-wall remodelling during the symbiotic interaction between *Tuber melanosporum* and *Corylus avellana* using a carbohydrate microarray

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Keywords: Carbohydrate-Active enZYmes, CoMPP, Ectomycorrhiza, Hazel, Plant cell wall, Tuber

Abbreviations

CBM: Carbohydrate-binding module

COMPP: Comprehensive microarray polymer profiling

GH: Glycoside hydrolase

HG: Homogalacturonan

PCWDEs: Plant cell-wall degrading enzymes

Introduction

Plant cell walls consist of a number of distinct biopolymers that are woven together into a complex network. They are the key determinants of plant shape, growth and development, and are collectively the largest source of biomass on earth. Although differing in macromolecular composition, plant cell walls are responsible for many of the same functions as animal extracellular matrixes, connecting cells into tissues, and they are involved in signalling cell growth and division (Lodish et al. 2000). Cell walls also constitute a plant's first line of defence against pathogens, and are an important feature that should be considered concerning the changes in cell wall composition during interactions with other organisms. However, plant cell walls are not only a substrate for the growth of fungi and a barrier to infection from parasites, they are also the first surface where contact with symbiotic fungi occurs (Balestrini and Bonfante 2014). In ectomycorrhizae, plant and fungal cell walls come into direct contact, and they represent the interface between the two partners. When the fungal hyphae penetrate the plant root cells, only subtle alterations can be observed in the plant cell walls, although a localized loosening has been reported in several ectomycorrhizal systems (Balestrini et al. 1996; Balestrini and Bonfante 2014).

It has recently been reported that the genomes of the ectomycorrhizal fungi *Laccaria bicolor* and *Tuber melanosporum* contain a reduced gene set involved in the production of enzymes that are active on plant cell-wall polysaccharides (Martin et al. 2008, 2010), in comparison with saprotrophic and pathogenic fungi (Martin and Selosse 2008). A reduced set of plant cell-wall degrading enzymes (PCWDEs) has also been reported for *Amanita bisporigena* by Nagendran et al. (2009), who showed that this ectomycorrhizal fungus lacks the genomic potential to biosynthesize many extracellular enzymes that are active on the linkages in the plant cell wall and, in particular, exhibits a loss of several key cellulase genes. A large-scale comparative genomics analysis of fungal genomes with different life-styles, including a dozen ectomycorrhizal fungi, has confirmed that the latter have a reduced complement of genes encoding PCWDEs, thus suggesting that, during their evolution, ectomycorrhizal fungi have lost much of the enzymatic apparatus of their ancestral wood decayers (Kohler et al. 2015). However, several species have retained a restricted set of PCWDEs, which seems to suggest that they still possess some PCW degradation and/or loosening ability (Kohler et al. 2015). In the past, enzymatic activities related to PCWDEs were demonstrated for ectomycorrhizal fungi, and several capacities in different fungal species were reported (Maijala et al. 1991; Cao and Crawford 1993; Colpaert and van Laere 1996; Kusuda et al. 2008). The *L. bicolor* genome contains a limited repertoire of enzymes putatively active on cellulose and hemicellulose, and it has recently been shown that they are expressed during the first steps of root cell colonization. On the other hand, the *L. bicolor* enzymes likely involved in pectin hydrolysis are mainly expressed at the fully mature ectomycorrhizal stage (Veneault-Fourrey et al. 2014). While the abovementioned studies have focused on the role of PCWDEs in ectomycorrhizae, the effect on plant cell walls during ectomycorrhizal fungal colonization still remains poorly studied and requires further efforts (Balestrini and Bonfante 2014 and references therein).

Although plant and fungal cell-wall components have been previously localized in ectomycorrhizal symbiosis (Balestrini et al. 1996), mapping the complex and diverse glycans in plant cell walls is a considerable technical challenge, mainly in a complex system such as an ectomycorrhiza where plant and fungal tissues are present. Several conventional biochemical techniques are available, but carbohydrate microarrays have emerged as a useful tool for high throughput semi-quantitative analysis. One technique, known as Comprehensive Microarray Polymer Profiling (CoMPP), has been used extensively to map defined glycan structures (epitopes) across large sample sets (Moller et al. 2007; Sørensen and Willats 2011). CoMPP involves the sequential extraction of cell wall polymers, which are printed as microarrays and probed with sets of cell wall-specific monoclonal antibodies (mAbs) and carbohydrate-binding modules

(CBMs) (Moller et al. 2007, 2008). CoMPP combines the high throughput capacity of microarray technology with the specificity of mAbs and CBMs, and allows glycan profiles to be rapidly generated in diverse systems. It has been used to study polysaccharide diversity across the plant kingdom (Fangel et al. 2012), and in different conditions, such as during interactions with other living organisms (Moller et al. 2011; Hansen et al. 2014; Johnsen et al. 2015). Recently, this approach has been applied in an elegant study aimed at understanding the interaction between the plant parasite *Cuscuta reflexa* and its host plant, starting from the idea that the compositional differences of cell walls could explain why some hosts are amenable to such degradation while others can resist infection (Johnsen et al. 2015).

Taking advantage of CoMPP technology, the current study is aimed at establishing the changes in plant cell wall composition in common hazel (*Corylus avellana*) roots during colonization by the ascomycetous ectomycorrhizal fungus *T. melanosporum*. This specific plant–fungus interaction has here been focused on as the hazelnut tree is a typical host plant for truffles, and in fact, it has also been used to analyse the *T. melanosporum* transcriptome during the symbiotic stage (Martin et al. 2010; Tisserant et al. 2011). Genes encoding putative PCWDEs were identified in the *T. melanosporum* genome, and previous large-scale transcriptomics data (Martin et al. 2010) of candidate genes were validated, using RT-qPCR, on *C. avellana*/*T. melanosporum* ectomycorrhizae.

Materials and methods

Biological materials

Fully developed *Tuber melanosporum* ectomycorrhizae and non-mycorrhizal root tips from the root systems of 1 year and a half old hazel plantlets (*Corylus avellana* L.), previously inoculated by a *T. melanosporum* mycelium slurry produced from a fruiting body (Martin et al. 2010), and grown in the AGRITRUFFE greenhouse facilities (Saint-Maixant, France) have been sampled under a stereomicroscope. A section of one ectomycorrhiza representative of those used in this work is shown in Supplementary Fig. S1, where it is possible to appreciate the presence of a considerable amount of fungal tissue. On the basis of the RNA proportion, it has been estimated that the amount of *Tuber* mycelium in ectomycorrhizal roots at this development stage is ~30 % (Tisserant et al. 2011). The *T. melanosporum* mycelium (Tmel28 strain) used for the molecular analysis was grown in a liquid modified Melin Norkrams (MMN) medium in a dark room at 25 °C for 1 month before harvesting.

Detection of putative PCWDEs encoded by Tuber melanosporum

The gene models (putative genes) corresponding to the proteins involved in plant cell-wall synthesis/degradation were identified at the TuberDB *Tuber* genome v. 1 database (<http://mycor.nancy.inra.fr/IMGC/TuberGenome/>). The best protein models from Tubme1 were compared with the sequences in the Carbohydrate Active Enzymes database (CAZy; www.cazy.org) (Lombard et al. 2014), and the proteins that generated a better *e* value than 0.1 were considered for a manual analysis. The latter step involved comparisons with the individual protein domains constitutive of CAZymes to eliminate false positives and assign the remaining proteins to the appropriate family (or families in the case of multiple CAZy modules). The sequences assigned to the glycosyl hydrolase (GH) GH5 and GH13 families

were further assigned to subfamilies, as described in Aspeborg et al. (2012) and Stam et al. (2006), respectively. Signal peptides were predicted using Phobius (Käll et al. 2004).

Functional annotation of putative PCWDEs

To carry out a functional prediction of the putative PCWDEs, a sequence library was built with only the sequences corresponding to CAZymes with published activity, as listed on the characterized pages of the CAZy database (for instance http://www.cazy.org/GH5_characterized.html for the GH5 family). This sequence library, which counts over 12,000 sequences, was interrogated with BLAST using the putative PCWDE sequences as queries. In other words, the aminoacid sequences of all the encoded GHs, polysaccharide lyases (PLs), carbohydrate esterases (CEs) and auxiliary activities (AAs) were used as queries against all the sequences of the experimentally characterized CAZymes derived from the CAZy database. Significant hits (with a better e value than 10^{-6}) with experimentally characterized PCWDEs were considered to be putative PCWDEs and retained for activity prediction. Activities were only considered predicted when the hits displayed at least 40 % sequence identity with the aminoacid sequence of a characterized PCWDE, to avoid problems due to the presence of multiple specificities in a given CAZY family.

RNA isolation and cDNA synthesis

Total RNA extraction from the mycelium and ectomycorrhizae was carried out using the 'pine tree method' (Chang et al. 1993). Genomic DNA was removed using a Turbo DNA-free™ reagent (Ambion, Austin, TX, USA), according to the manufacturer's instructions. The cDNA synthesis has been described in detail in Balestrini et al. (2012).

Gene expression analysis

Large-scale transcriptomic data were previously obtained from a *T. melanosporum* custom-exon expression array (4 × 72 K) manufactured by Roche Nimblegen Systems Limited (Madison, WI, USA) and from massive cDNA sequencing, with Illumina technology, of the samples of the free living mycelium and ectomycorrhizae, as described in detail in Martin et al. (2010). Quantitative RT-PCR (RT-qPCR) experiments were conducted to validate the large-scale transcriptomic results. Specific primers were designed for the considered genes (Table S1) using PerlPrimer software v 1.1.19 (<http://perlprimer.sourceforge.net/>) and verified by means of Primer BLAST Software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>). Before RT-qPCR, the primers were tested on genomic DNA from *T. melanosporum* mycelium (as a positive control) and *C. avellana* leaves (as a negative control). Genomic DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). To assess the specificity of the primers, RT-PCR on cDNA from *T. melanosporum* mycelium was performed as described in Sillo et al. (2015) and amplified products were sequenced. Purification of products was performed using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) at 37 °C for 15 min and subsequently at 80 °C for 15 min. The purified products were sequenced at BMR Genomics S.R.L. (Padua, Italy). Each sequence was aligned with the coding sequence of its gene model using MEGA v. 6.0 (Tamura et al. 2013).

Quantitative RT-PCR was carried out with StepOne apparatus (Applied Biosystems, Foster City, CA, USA). Each PCR was performed in a volume of 20 μ l containing 10 ng cDNA (1 μ l), 10 μ l 2 \times SYBR[®] Green PCR Master Mix (Applied Biosystems) and 2 μ l of each primer (3 μ M stock) using a 48-well plate. The following PCR programme, which includes the calculation of a melting curve, was used: 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 for 1 min, 95 °C for 15 s. The baseline range and CT values were automatically calculated using the StepOne software. The expression of candidate genes was normalized to that of TmeIEF1B (Sillo et al. 2013), which was used as the housekeeping gene, by subtracting the CT value of TmeIEF1B from the CT value of the candidate gene resulting from the Δ CT. The expression ratio was calculated, without the PCR efficiency correction, from Eq. 2— $\Delta\Delta$ CT, where $\Delta\Delta$ CT represents the Δ CT_{sample} – Δ CT_{control}. All the reactions were performed for two biological and three technical replicates. Statistical analyses were carried out using Rest2008, version 2.0.7 (Pfaffl et al. 2002), considering data with a P value <0.05 as being significantly different.

Cell wall material preparation

The cell wall material for the CoMPP analysis was prepared from ectomycorrhizae and non-mycorrhizal root tips as an alcohol-insoluble residue (AIR). Samples were collected carefully from the hazelnut root system using a sterile scalpel and tweezers, and placed in 2-ml tubes. The collected samples were dry-lyophilized overnight and homogenized into powder using Tissue Lyser (5 min, 28 Hz). Subsequently, the samples were re-suspended in 70 % ethanol for 30 min at room temperature under rotation. The supernatant was removed after centrifugation at 22,000g for 10 min. This step was performed five times. The time of suspension in 70 % ethanol was increased to 60 min for the last two steps. A final wash was performed with 100 % acetone, and an AIR pellet was recovered after a final centrifuge at 22,000g for 15 min. The AIR pellet was dried at room temperature.

Comprehensive microarray polymer profiling

CoMPP was carried out as described in Moller et al. (2007). Cell wall polymers were extracted sequentially from 10 mg of AIR with 300 μ l of 50 mM diamino-cyclo-hexane-tetra-acetic acid (CDTA), pH 7.5 and 4 M NaOH with 0.1 % v/v NaBH₄ and spotted on a nitrocellulose membrane with a pore size of 0.45 μ m (Whatman, Maidstone, UK) using an Arrayjet Sprint (Arrayjet, Roslin, UK). Each sample was printed with two technical replicates and four dilutions, and probed as described in Pedersen et al. (2012). The arrays were scanned using a flatbed scanner (CanoScan 8800 F, Canon, Søborg, Denmark) at 2400 dpi and quantified using Array-Pro Analyzer 6.3 (Media Cybernetics, Rockville, MD, USA). The error bars represent the SE values of six independent extractions.

Immunogold labelling

Immunogold experiments were performed as described in Balestrini et al. (1996). Monoclonal antibodies (mAbs) JIM5 and JIM7 (Knox et al. 1990; Verhertbruggen et al. 2009; Carbon Source, Athens, GA, USA) were used (1:10 dilution) to localize homogalacturonan (HG) with a different esterification degree (JIM5 recognizes partially methyl-esterified HG epitopes, including un-esterified HG; JIM7 recognizes more methyl-esterified HG epitopes, and does not bind to un-esterified HG), while an mAb anti- β -1,3-glucan

(Biosupplies, Parkville, Australia) was used to localize glucans (1:100 dilution). The primary antibody was omitted for the negative control. Thin sections (0.05–0.07 μm) were post-stained with uranyl acetate and lead citrate before being observed under a Philips CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Results

Catalogue of genes coding PCWDEs in T. melanosporum

The several GHs, PLs and CEs have been identified in the *T. melanosporum* genome. The manual annotation and activity prediction of genes coding PCWDEs are reported in Table 1. Genes coding for cellulolytic enzymes such as the proteins of the GH5_5, GH12, GH45 (endoglucanases) and AA9 (lytic polysaccharide mono-oxygenases that oxidatively cleave cellulose) families have been found, while the cellobiohydrolases of the GH6 and GH7 families were absent from the *T. melanosporum* genome. Pectin degradation enzymes are represented by members of the PL1 (pectin/pectate lyases), PL4 (rhamnogalacturonan lyase), CE12 (rhamnogalacturonan acetyltransferase), GH28 (polygalacturonases), GH43 (α -L-arabinanase), GH78 (α -L-rhamnosidases) and CE8 (pectin methyltransferase) CaZy families. Only one putative xylanase (GH10 family) appears to be encoded by the *T. melanosporum* genome. Most of the PCWDEs appear to have a secretion signal peptide (Table 1), and a lytic polysaccharide monooxygenase (LPMO) of the AA9 family (JGI ID = 3661) carries a cellulose-binding domain (CBM1) module.

Table 1 Putative plant cell-wall degrading enzymes (PCWDEs) in *T. melanosporum* genome

Substrate	Gene model#	Gene name	CAZy family	Predicted activity	Signal peptide	JGI protein ID
Cellulose	GSTUMT00008973001	<i>TmelCMC3</i>	GH5_5	Endoglucanase	No	5467
	GSTUMT00008986001	<i>TmelLPMO1</i> [^]	AA9	LPMO*	Yes	5474
	GSTUMT00001211001	<i>TmelLPMO2</i> [^]	AA9	LPMO*	No (fragment too short at N-term)	3661
	GSTUMT00009298001	<i>Tmelcel</i>	GH12	Endoglucanase	Yes	5666
	GSTUMT00011089001	<i>TmelEG</i>	GH45	Endoglucanase	Yes	6760
	Pectin	GSTUMT00008534001	<i>TmelPLB</i>	PL1	Pectin/pectate lyase	Yes
GSTUMT00006239001		<i>TmelplA</i>	PL1	Pectin/pectate lyase	No (fragment too short at N-term)**	3478
GSTUMT00004952001		<i>TmelrghB</i>	PL4	Rhamnogalacturonan lyase	Yes	1834
GSTUMT00003723001		<i>TmelRgaE</i>	CE12	Rhamnogalacturonan acetyltransferase	Yes	2410
GSTUMT00012011001		<i>TmelPGN1</i>	GH28	Polygalacturonase	Yes	7339
GSTUMT00004199001		<i>TmelrghA</i>	GH28	Polygalacturonase	Yes	268
GSTUMT00012789001		<i>Tmelpme1</i>	CE8	Pectin methyltransferase	Yes	–
GSTUMT00003229001		<i>TmelabnA</i>	GH43	α -L-arabinanase	Yes	1920
GSTUMT00005050001		<i>Tmelrha1</i>	GH78	α -L-rhamnosidase	Yes	2086
GSTUMT00006913001		<i>Tmelrha2</i>	GH78	α -L-rhamnosidase	No	4095
Hemicellulose		GSTUMT00009198001	<i>TmelxlnA</i>	GH10	xylanase	No

[^] LPMO lytic polysaccharide mono-oxygenase active on cellulose

** In *Tuber melanosporum* Mel28 v2 is complete and has a signal peptide

[^] Respectively *Tmeleg11* and *Tmeleg12* in Tuber DB (<http://mycor.nancy.inra.fr/IMGCTuberGenome/index.html>)

Validation of large-scale transcriptome data

The expression of PCWDEs in *T. melanosporum* was carried out for three growth conditions (ectomycorrhizal root tips; fruiting bodies and free-living mycelium) using RNA-Seq and cDNA arrays, as described in Martin et al. (2010). These analyses have shown that several of these genes are regulated

during the symbiotic stage (Martin et al. 2010; Table 2). The complete expression data set is available as a series (accession number GSE17529) in the Gene Expression Omnibus at NCBI (<http://www.ncbi.nlm.nih.gov/geo/>). According to the cDNA array results, 8 out of 16 genes encoding putative PCWDEs were more than 4-fold up-regulated in the *T. melanosporum* ectomycorrhiza vs. the free-living mycelium (FLM) (Table 2). Here, nine gene models coding for enzymes putatively acting on cellulose (i.e., TmelCMC3, TmelLPMO1, Tmelcel, TmelEG) and on pectin (i.e., TmelPLB, TmelRgaE, TmelPGN, TmelrghA, Tmelpme1) were considered in quantitative RT-PCR (RT-qPCR) reactions to evaluate their expression in fully established ectomycorrhizae compared to free-living mycelium. As reported in Table 2, the results confirmed the array trend for almost all of the considered genes, although with some differences. In other words, RT-qPCR experiments showed a high up-regulation of a gene coding for a putative rhamnogalacturonan acetyltransferase (TmelRgaE) that appeared only slightly regulated in the array (although with a non-significant P value >0.05), and a gene coding for an AA9 (TmelLMPO1), which was only weakly up-regulated in the array, also resulted to be highly up-regulated. Unlike for the array data, a gene coding for a putative pectin/pectate lyase (TmelPLB) also resulted to be up-regulated, in agreement with the RNAseq data.

Table 2 Expression values in RT-qPCR (\pm SD), microarray and RNAseq analyses

Gene name	Blastp (best identified match)	Organism	Query coverage (%)	<i>e</i> value	Identity (%)	Fold change RT-qPCR	Array ratio ECM/FLM	RNAseq ECM (RPKM)	RNAseq FLM (RPKM)
<i>TmelCMC3</i>	Similar to Probable endo-beta-1,4-glucanase B	<i>Pyronema omphalodes</i>	92	1e-171	75	143.83 \pm 16.97	6212.62	245.2	0.0
<i>TmelLPMO1</i>	Similar to Endoglucanase-4	<i>Pyronema omphalodes</i>	57	2e-81	61	272.24 \pm 19.36	2.64	570.6	45.9
<i>Tmelcel</i>	Similar to Xyloglucan-specific endo-beta-1,4 glucanase A	<i>Pyronema omphalodes</i>	99	8e-87	57	199.62 \pm 11.47	225.22	71.5	1.8
<i>TmelEG</i>	Endoglucanase-5	<i>Pyrenophora tritici-repentis</i>	100	4e-100	62	14.34 \pm 15.38	14.65	24.0	2.3
<i>TmelPLB</i>	Putative pectate lyase	<i>Neofusicoccum parvum</i>	95	8e-131	71	23.86 \pm 14.56	0.07*	60.3	3.9
<i>TmelRgaE</i>	Similar to Rhamnogalacturonan acetylsterase	<i>Pyronema omphalodes</i>	87	1e-142	66	224.72 \pm 12.06	4.44*	83.4	6.4
<i>TmelPGN1</i>	Putative endopolygalacturonase	<i>Diplodia seriata</i>	97	4e-146	62	22.91 \pm 24.83	29.46	244.2	16.4
<i>TmelrghA</i>	Putative endo-rhamnogalacturonase f	<i>Phaeoemiella chlamyospora</i>	79	2e-154	58	4.72 \pm 11.00*	4.63	18.9	4.0
<i>Tmelpme1</i>	Carbohydrate esterase family 8 protein	<i>Fomitiporia mediterranea</i>	64	7e-72	41	4.00 \pm 1.10	31.87	37.7	4.1
<i>TmelLPMO2</i>	Putative endo-beta-1,4-glucanase D	<i>Glarea lozoyensis</i>	93	8e-65	47	–	1.50*	48.1	0.9
<i>TmelpnlA</i>	Putative pectin lyase a precursor	<i>Diplodia seriata</i>	99	1e-116	55	–	2.89*	8.6	2.7
<i>TmelrghB</i>	Rhamnogalacturonan lyase	<i>Marssonina brunnea</i>	99	0.0	61	–	2.48*	0.0	0.3
<i>TmelabnA</i>	Arabinan endo-1,5-alpha-L-arabinosidase A	<i>Pestalotiopsis fici</i>	99	3e-137	64	–	1.82*	17.5	16.3
<i>Tmelrha1</i>	Alpha-l-rhamnosidase a	<i>Colletotrichum orbiculare</i>	93	0.0	44	–	0.86*	20.4	11.8
<i>Tmelrha2</i>	Alpha-L-rhamnosidase	<i>Streptomyces xylophagus</i>	91	0.0	54	–	1.12*	3.0	1.5
<i>TmelxlnA</i>	Glycoside hydrolase family 10 protein	<i>Hydnomerulius pinastri</i>	99	3e-131	61	–	0.08*	10.4	23.3

Microarray ratio and RPKM values were obtained from Martin et al. (2010). Genes that are not significantly differentially regulated are marked with the asterisk (*P* value >0.05) ECM Ectomyconhizae, FLM Free-living mycelium, – not analyzed in RT-qPCR, RPKM reads per kb per million mapped reads

Mapping of the plant cell wall polymers in *Corylus avellana* roots and in ectomycorrhizae

Cell-wall polysaccharides were sequentially extracted from non-mycorrhizal roots and ectomycorrhizae using CDTA and NaOH solvents. The two bar diagrams that can be observed in Fig. 1 show the mean CoMPP signals obtained for each mAbs. Combining these data across the two extractions, an overview of the changes was obtained in the cell-wall epitope levels in ectomycorrhizae, in comparison with non-colonized hazel roots. Considering non-colonized roots, the CDTA extraction, which mainly solubilises pectins, shows signals for HG, as revealed by the mAbs JIM5, JIM7, LM18, LM19, and LM20, and arabinogalactan proteins (JIM13), while the main epitopes detected in the NaOH extract are from hemicellulosic mannans (BS-400-4 and LM21), xyloglucans (LM15 and LM25) and xylans (LM10, LM11 and LM23). In the CDTA extract from ectomycorrhizae, the HG content is reduced to a great extent, as shown by the JIM5, JIM7, LM18, LM19, and LM20 signals. In the NaOH extract, xyloglucans (LM15) and xylans (LM10, LM11, and LM23) are clearly decreased, while the remaining polysaccharides show reductions of smaller magnitude (Fig. 1). In general, the analysis shows that the fungal colonization affects most of the polymer groups. However, due to the presence of cell wall material of fungal origin in the ectomycorrhizal samples (Fig. S1), the observed reduction in all the cell wall polysaccharide groups could also result from a dilution of plant material.

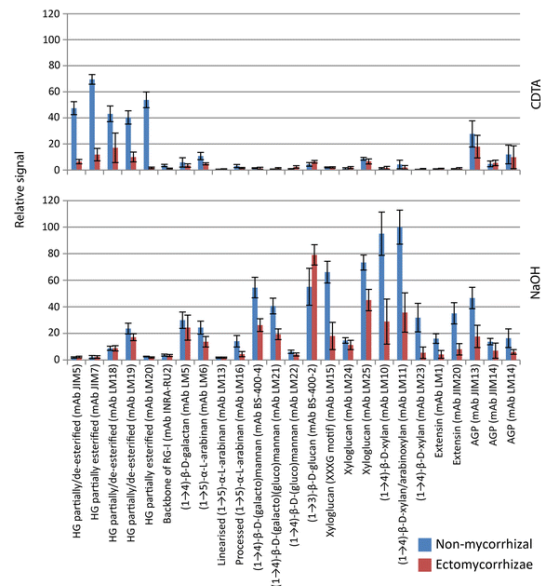


Fig. 1

CoMPP analysis of ectomycorrhizal and non-mycorrhizal cell-wall fractions showing the relative abundance of specific cell-wall polysaccharides found in pectin (CDTA) and hemicellulose (NaOH) extractions. Spot intensities were quantified and the highest value in the dataset was set to 100 and all the other data adjusted accordingly. Each value is the average of the mean spot signals from two experiments each involving 3 biological replicates

Localization of homogalacturonan in *C. avellana*/*T. melanosporum* ectomycorrhizae

Labelling experiments have been performed to localize different methyl-esterified HG on sections of *C. avellana* ectomycorrhizae and non-colonized roots. When JIM5, which recognizes partially methyl-esterified HG epitopes including un-esterified HG, was used on non-colonized root sections, abundant gold granules appeared on the outer surface of the epidermal cells, on junctions between cells, and on the middle lamella (not shown), in agreement with previous observations (Balestrini et al. 1996). In ectomycorrhizae, fungal hyphae develop among the cortical root cells to form the Hartig net region (Fig. 2a). After treatment with JIM5, gold granules were present on the cell wall surrounding the fungal hyphae in close contact with the fungal cell walls (Fig. 2b, c), as well as in the regions without the fungus, on the middle lamella and on the

junctions between three host cells (Fig. 2d), as in the non-colonized roots (not shown). The presence of some gold granules in the mantle region is due to collapsed epidermal cells being entrapped among the mantle hyphae, whose external cell walls were labelled, after treatment with JIM5, on non-colonized root sections (not shown). However, only a few gold granules were evident on the plant cell walls (regardless of the cell-type) when mAb JIM7, which recognizes more methyl-esterified HG epitopes, was used, both in the ectomycorrhiza (Fig. 2e) and non-colonized roots (not shown).

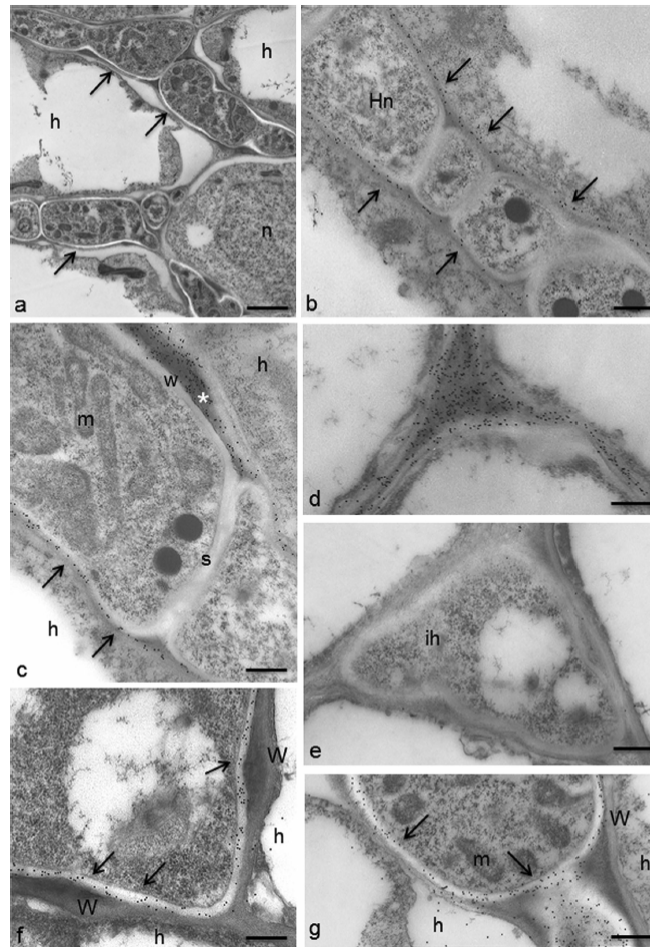


Fig. 2 JIM5 (a–d), JIM7 (e) labelling to localize homogalacturonans and anti- β -1,3-glucan (f, g) labelling on ultra-thin sections of *C. avellana*/*T. melanosporum* ectomycorrhiza. a Ultrastructure view of the Hartig net region (arrows). A full set of intracellular organelles is visible within the fungal hyphae. b After treatment with JIM5, homogalacturonan labelling (arrows) is present on the cell wall of the cortical cells bordering the Hartig net (Hn) hyphae, while the fungal walls and the electron-dense triangle are not labelled. c Higher magnification of the plant–fungus contact zone in the Hartig net region. Gold granules are present only on the host cell walls (arrows), and consistent labelling is visible on the middle lamella residues (asterisk) attached to the fungal cell wall. d JIM5 labelling of homogalacturonans on non-colonized root cell walls. Evident labelling is present at a junction between cortical cells. e After treatment with anti-homogalacturonan JIM7 antibodies on ultra-thin sections of an ectomycorrhiza, only a very few gold granules are present on the plant cell walls bordering an intercellular hypha (ih). f, g Localization of β -1,3-glucans in *C. avellana*/*T. melanosporum* ectomycorrhiza. Abundant labelling (arrows) is present on the cell walls of hyphae penetrating the host cells (f) and in the Hartig net region (g). h host cell, Hn Hartig net, m mitochondria, n nucleus, s septum, w fungal

cell wall, W plant cell wall. Scale bars 1.5 μm (a), 0.56 μm (b), 0.47 μm (c), 0.43 μm (d), 0.39 μm (e), 0.36 μm (f), 0.48 μm (g)

Discussion

Plant cell wall degrading enzymes in T. melanosporum

CAZyme (Carbohydrate Active enZymes) annotation has been performed in the frame of *T. melanosporum* genome project (Martin et al. 2010) and the putative plant cell wall degrading enzymes (PCWDEs) have been identified. Since the last CAZyme annotation of *T. melanosporum* dates back to the genome sequencing project (Martin et al. 2010), an update of the annotation was performed to take into account the increased number of reference sequences and their improved annotations in the CAZy database (Lombard et al. 2013). The results have confirmed the presence of a reduced set of genes coding PCWDEs, as previously reported (Martin et al. 2010). This is a common feature of all ectomycorrhizal fungi (Veneault-Fourrey et al. 2014; Kohler et al. 2015) as well as of brown-rot fungi and the biotrophic fungus *Melampsora larici-populina* (Duplessis et al. 2011). Enzymes targeting all of the three components of plant cell wall polysaccharides (cellulose, pectin, hemicellulose) have been identified in the *T. melanosporum* genome, because of their close relationship with biochemically characterized CAZymes. Cellulolytic activities were represented by proteins of the GH5_5, GH12, GH45 (endoglucanases) and AA9 (lytic polysaccharide mono-oxygenases that oxidatively cleave cellulose) families. It should be noted that the cellobiohydrolases of the GH6 and GH7 families, which intervene in the extensive saccharification of cellulose, were absent from the *T. melanosporum* genome. In agreement with the known complexity of the substrate (Willats et al. 2006), pectin degradation enzymes were the most numerous in the *T. melanosporum* genome, with the members of the PL1 (pectin/pectate lyases), PL4 (rhamnogalacturonan lyase), CE12 (rhamnogalacturonan acylesterase), GH28 (polygalacturonases), GH43 (α -L-arabinanase), GH78 (α -L-rhamnosidases) and CE8 (pectin methylesterase) CAZy families. On the other hand, only one putative xylanase (GH10 family) appears to be encoded by the genome.

Fungal PCWDEs often show a modular structure with CBM1 family non-catalytic modules that target the enzyme appended to cellulose (Levasseur et al. 2013). Although saprotrophic fungi usually have several CBM1-containing proteins (sometimes several dozen), the ectomycorrhizal fungus *L. bicolor* contains a single CBM1, appended to a GH5 endoglucanase (Martin et al. 2008), and the *T. melanosporum* genome appears to encode only three proteins that carry a CBM1 module: an LPMO of the AA9 family (JGI ID = 3661; TmelLPMO2), a protein related to tyrosinase (JGI ID = 5167) and a protein of an unknown function (JGI ID = 3974). This result is also in agreement with the recent data which show that proteins with a cellulose-binding domain (CBM1) are rarely detected in ectomycorrhizal fungal genomes, while they are abundant in the orchid and ericoid mycorrhizal fungal symbionts (Kohler et al. 2015).

Expression of T. melanosporum PCWDEs during symbiosis

Less information is available on the colonization mechanisms of symbiotic fungi than on the hydrolytic activities of plant pathogenic microbes and fungi (Kubicek et al. 2014). Recently, genome-wide transcriptome profiling, during ectomycorrhiza development, has been used to explore the *L. bicolor*

CAZome (i.e., the collection of carbohydrate-active enzymes encoded by the genome of an organism) during symbiosis establishment (Veneault-Fourrey et al. 2014). Here, RT-qPCRs were performed to validate the array data that originated in the frame of the *T. melanosporum* genome project (Martin et al. 2010), and the results confirmed the array trend of almost all of the considered genes (Table 2). As already reported in Balestrini et al. (2012), differences in gene expression data obtained using different techniques are generally present when the array data are not significant (with a P value >0.05), but they can also be explained considering the variability in the biological material sampling methods. Fully developed ectomycorrhizae are collected from plants grown in pots, but they might not be at exactly the same developmental stage. The expression trend of genes coding for enzymes acting on pectin, such as two GH28 (TmelPGN1 and TmelrghA) and a CE8 (Tmelpme1), has here been confirmed. In comparison with the array data, the quantitative RT-PCR data have shown the up-regulation of an additional gene putatively involved in pectin degradation, TmelPLB, coding for a protein that shows homology with a pectate lyase, in agreement with the RNAseq data. Gene coding enzymes that could act on cellulose, such as a gene coding for a GH5 (TmelCMC3), which is one of the most highly up-regulated genes in ectomycorrhizae, and two genes coding for GH12 (Tmelcel) and GH45 (TmelEG), respectively (Table 2), have also been up-regulated in truffle ectomycorrhizae. Interestingly, TmelCMC3 (GH5) was more expressed in the Hartig net compartment, thus suggesting a role during fungal penetration (Hacquard et al. 2013). In addition, it has been found that a gene coding for AA9 (TmelLPMO1) and a gene coding for a putative rhamnogalacturonan acetyltransferase (TmelRgaE) are strongly up-regulated, while only a slight up-regulation has been detected from the cDNA arrays. The regulation of these genes, with an up-regulation in the ectomycorrhizal tissues, suggests their involvement when the fungus penetrates the host cells and the host walls are separated, which would allow fungal root colonization. These data are in agreement with recent data on young *T. melanosporum* ectomycorrhizae that have shown an overexpression of several glycoside hydrolase genes versus the free-living mycelium, thus reflecting the ability to degrade host cell walls (Le Tacon et al. 2015). It has also been reported that two *T. melanosporum* transcription factor (TF) genes encoding homologs of XlnR, an activator of cellulose/hemicellulose degrading enzymes in other fungi (Aro et al. 2005), are up-regulated in *T. melanosporum* ectomycorrhizae (Montanini et al. 2011). It has recently been suggested that the ectomycorrhizal fungus *L. bicolor* regulates PCWDE expression, thus allowing a “gentle” plant cell-wall remodelling during plant colonization (Veneault-Fourrey et al. 2014). The possibility of performing these analyses at different stages of the interactions has made it possible to demonstrate that the *L. bicolor* CAZymes involved in hemicellulose degradation are mainly expressed during mantle formation and the root penetration of the first epidermal cells, while the CAZymes that are active on pectin and cellulose are expressed more in developed ectomycorrhizae (Veneault-Fourrey et al. 2014).

Moreover, a laser microdissection approach to dissect the response in the two fungal compartments in *T. melanosporum* ectomycorrhizae has made it possible to verify the concomitant induction of *T. melanosporum* genes coding for endoglucanase (GH45), an LPMO (AA9) and a cellodextrin transporter at the soil/mantle interface, thus suggesting the ability of *T. melanosporum* to weakly degrade cellulose (i.e., extracellular cellulolytic activity) and to assimilate the products of this activity (Hacquard et al. 2013).

Hazelnut cell wall composition in the presence and absence of the ectomycorrhizal fungus

To investigate the changes related to root colonisation by the symbiotic fungus at the biochemical level, CoMPP (Moller et al. 2007) was used to measure the intensity with which antibodies recognize specific epitopes in the cell walls of ectomycorrhizal and non-mycorrhizal root tips (Fig. 1). The CoMPP profile of the

non-mycorrhizal roots points to a typical cell wall pattern for angiosperms (Moller et al. 2007; Fangel et al. 2012), while a decrease in all of the cell wall polymers has been revealed in the ectomycorrhizal sample, except for (1 → 3)-β-d-glucan, which can be assigned to the fungus, which also contains this polymer (Balestrini et al. 2012; Fig. 2). Although a reduction in these polymers, due to the presence of the fungal cell wall material, which could lead to a dilution of the material of plant origin, cannot be excluded, these results are in agreement with the fact that, during the colonization process, the fungus penetrates the root cells through the pectin-rich middle lamella and occupies the intercellular spaces of the root cortex (Fig. 2). The up-regulation of GH12 could explain the decrease in xyloglucans, as demonstrated by the CoMPP analysis, considering the xyloglucan specificity displayed by certain GH12 enzymes (Damásio et al. 2014). The fact that the BLASTp analysis has pointed out that Tmelcel shows homology (e value: 1e-74, identity: 56 %) with an *Aspergillus oryzae* protein (XP_001820024.1; gene name, xgeA) described as a probable xyloglucan-specific endo-beta-1,4-glucanase A (EC:3.2.1.151) (Machida et al. 2005) supports this hypothesis.

Labelling experiments on *C. avellana* and *T. melanosporum* ectomycorrhizal sections has been performed to localize pectin compounds, and they have shown a different localization pattern in ectomycorrhizae for HG with a low density of methyl esterification, as recognized from the JIM5 monoclonal antibody, compared to the non-colonized roots. These results are in agreement with a previous work on hazel ectomycorrhizae, but for a different truffle species (Balestrini et al. 1996). The fact that no labelling has been seen for JIM 7 (which recognizes partially methyl-esterified HG but does not bind to un-esterified HG), although a signal for this compound is present in the CoMPP analysis, could suggest low accessibility of this polymer to the hazelnut cell wall. On the other hand, the fact that *T. melanosporum* cell walls are always labelled after treatment with the anti-β-1,3-glucan monoclonal antibody, as previously reported (Balestrini et al. 2012), suggests that the presence of higher signal for this component in the ectomycorrhizae in the CoMPP analysis can be correlated to its presence in the fungal cell wall.

Conclusions

CoMPP approach has been used here for the first time to detect changes in plant cell wall composition during an ectomycorrhizal interaction, thus confirming that the plant cell wall undergoes modification during the symbiotic stage. Although our data underline a difficulty in the interpretation of the results due to the presence of both fungal and plant cell-wall components, a localized degradation of HG (the most abundant plant pectin polymer) can be suggested during fungal colonization, in agreement with the growth of the colonizing fungus through the middle lamella and the fungal gene expression of genes acting on these polysaccharides. Understanding HG remodelling by specific fungal or plant enzymes, and how these changes are spatially and temporally mediated, is a central issue in addressing the fundamental role of HG-type pectins during plant growth and in plant response to environmental changes (Sénéchal et al. 2014). Considering the role of de-methylesterified HG-derived oligogalacturonides (OGs) as signalling molecules (Ferrari et al. 2013), the present findings raise the question of whether HG hydrolysis can act as a source of pectin fragments that are capable of eliciting plant responses during ectomycorrhizal symbiosis. Direct evidence of OGs acting as *in vivo* elicitors of the plant defence response has recently been reported by Benedetti et al. (2015), who have shown that OG release and accumulation in planta confers resistance to pathogen attack.

On the other hand, de-methylesterified HG can form the so-called “egg box” structures through binding with Ca²⁺. It has been suggested that a reduced content of egg-box structures could make other

compounds, such as cellulose and hemicellulose, more accessible to their degrading enzymes (Lionetti et al. 2009). Other results obtained using a CBH-I complex to localize cellulose have suggested that a truffle species does not have an evident impact on this compound in the cell wall of hazel (Balestrini et al. 1996), although cell wall loosening has been suggested. The involvement of *T. melanosporum* enzymes acting on cellulose in cell wall loosening during fungal penetration can be hypothesized. A further step, which would probably require the development of an in vitro system, could be to evaluate the gene expression of the *T. melanosporum* PCWDEs at different points of time during the formation of ectomycorrhizae.

The forthcoming release of the genome sequences and annotations of additional Tuber species will allow comparison to be made of PCW degradation mechanisms in various ectomycorrhizal interactions. Finally, owing to the large variability of plant cell wall composition, it is possible that the host plant cell wall composition could influence the expression of fungal PCWDEs, and it could be interesting to investigate gene expression in several fungus/plant combinations.

Author contribution statement

FS conducted the gene expression experiments and contributed to writing the manuscript. JUF and WGTW conducted the CoMPP experiment and contributed to writing the manuscript. BE conducted the annotation and contributed to writing the manuscript. AF conducted the sample preparation for electron microscopy. PB critically revised the manuscript. FM coordinated the truffle genome project, provided the oligoarray datasets and critically revised the manuscript. RB planned the experiments, contributed to the gene annotation, conducted the microscopic observations and drafted the manuscript. All the authors read and approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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