Fungal Genetics and Biology 112 (2018) 47–54

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Regular Articles

Temporal transcriptome analysis of the white-rot fungus *Obba rivulosa* shows expression of a constitutive set of plant cell wall degradation targeted genes during growth on solid spruce wood

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ARTICLE INFO

Keywords:
Obba rivulosa
White-rot
Lignocellulose
Solid state cultivation
Transcriptomics

ABSTRACT

The basidiomycete white-rot fungus *Obba rivulosa*, a close relative of *Gelatoporia* (*Ceriporiopsis*) *subvermispora*, is an efficient degrader of softwood. The dikaryotic *O. rivulosa* strain T241i (FBCC949) has been shown to selectively remove lignin from spruce wood prior to depolymerization of plant cell wall polysaccharides, thus possessing potential in biotechnological applications such as pretreatment of wood in pulp and paper industry. In this work, we studied the time-course of the conversion of spruce by the genome-sequenced monokaryotic *O. rivulosa* strain 3A-2, which is derived from the dikaryon T241i, to get insight into transcriptome level changes during prolonged solid state cultivation. During 8-week cultivation, *O. rivulosa* expressed a constitutive set of genes encoding putative plant cell wall degrading enzymes. High level of expression of the genes targeted towards all plant cell wall polymers was detected at 2-week time point, after which majority of the genes showed reduced expression. This implicated non-selective degradation of lignin by the *O. rivulosa* monokaryon and suggests high variation between mono- and dikaryotic strains of the white-rot fungi with respect to their abilities to convert plant cell wall polymers.

1. Introduction

Plant biomass, as the most abundant renewable carbon source on Earth, is important not only for carbon cycling, but also as a feedstock for biofuels and newly derived value-added products (Isikgor and Becer, 2015). The main polymeric components comprising the plant cell wall, i.e. cellulose, hemicellulose, lignin and pectin, are responsible for its structural complexity. However, recalcitrance of lignocellulose is mostly due to the amorphous aromatic polymer lignin and presents the biggest obstacle in biotechnological exploitation of plant biomass.

Although a variety of microorganisms can attack lignocellulose, white-rot basidiomycete fungi are the most effective plant cell wall degrading organisms as they efficiently decompose all lignocellulose components by a variety of extracellular enzymes (Hatakka and Hammel, 2011; Mäkelä et al., 2014). Major cell wall polymers are being degraded by action of extracellular hydrolytic and oxidative enzymes, most of which have been categorized in the database of Carbohydrate-Active EnZymes (CAZy, http://www.cazy.org/) (Lombard et al., 2013). The resulting monomeric sugars are taken up by the fungal cells and metabolized as carbon and energy sources through specific pathways

Abbreviations: AA, auxiliary activity; AAO, aryl alcohol oxidase; AE, acetylesterase; AGL, α-galactosidase; AGU, α-glucuronidase; AOX, alcohol oxidase; BGL, β-1,4-glucosidase; CAZy, CAZyme, carbohydrate-active enzyme; CBH, cellobiohydrolase; CDH, cellobiose dehydrogenase; CE, carbohydrate esterase; CRO, copper radical oxidase; EGL, β-1,4-endoglucanase; ENO, enolase; FBA, fructose-bisphosphate aldolase; FET, ferroxidase; FPKM, Fragments Per Kilobase of exon model per Million fragments mapped; GAL, β-1,4-endogalactanase; GE, 4-O-methyl-glucuronyl methylesterase, glucuronoyl esterase; GH, glycoside hydrolase; GLX, glyoxal oxidase; GMC, oxidoreductase glucose-methanol-choline oxidoreductase; GND, 6-phosphogluconate dehydrogenase; GOX, glucose (1-)oxidase; GPD, glyceraldehyde-3-phosphate dehydrogenase; GT, glycosyl transferase; ICL, isocitrate lyase; LAR, L-arabinose reductase; LCC, laccase; LiP, lignin peroxidase; LN-AS, low nitrogen-asparagine-succinate; LPMO, lytic polysaccharide monooxygenase; MAN, β-1,4-endomannanase; MB, mega base pairs; MDH, malate dehydrogenase; MEA, malt extract agar; MND, β-1,4-mannosidase; MnP, manganese peroxidase; OXA, oxaloacetase; PCA, principal component analysis; PCP, pentose catabolic pathway; PCWDE, plant cell wall degrading enzyme; PFK, fructose-2,6-bisphosphatase; PGA, endopolygalacturonase; PGI, glucose-6-phosphate isomerase; PKI, pyruvate kinase; PL, polysaccharide lyase; PPP, pentose phosphate pathway; qRT-PCR, quantitative real-time PCR; RNA-seq, RNA sequencing; TAL, transaldolase; TCA cycle, tricarboxylic acid cycle; VP, versatile peroxidase; XDH, xylitol dehydrogenase; XG-EG, xyloglucanase, xyloglucan-active endoglucanase; XLN, β-1,4-endoxylanase

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http://dx.doi.org/10.1016/j.fgb.2017.07.004
Received 31 May 2017; Received in revised form 10 July 2017; Accepted 13 July 2017
Available online 25 July 2017
1087-1845/

(Khosravi et al., 2015).

Lignin degradation is a prerequisite for gaining access to carbohydrate polymers, which serve as a carbon and energy source for fungi (Rytioja et al., 2014). White-rot fungi produce an array of oxidoreductases from the families of auxiliary activities (AA) that are known to take part in lignin modification and degradation. Of those, the key enzymes are fungal class II peroxidases, i.e. lignin peroxidases (LiPs), manganese peroxidases (MnPs) and versatile peroxidases (VPs) that are present in all efficient lignin degrading white-rot fungi in different numbers. In addition, laccases that are phenol-oxidizing multicopper oxidases are suggested to participate in lignin conversion with peroxidases in the presence of the aromatic mediator molecules (Zhao et al., 2016). Moreover, several extracellular H_2O_2-generating enzymes are a part of ligninolytic system (Ferreira et al., 2015). These include glucose-methanol-choline (GMC) enzymes alcohol oxidases (AOXs), aryl alcohol oxidases, (AAOs) glucose 1-oxidases (GOXs), and copper radical oxidases (CROs) such as glyoxal oxidases (GLXs). White-rot fungi are able to completely depolymerize the plant cell wall polysaccharides by secreting various hydrolytic enzymes, including cellulases and hemicellulases, from several glycoside hydrolase (GH) families (Rytioja et al., 2014). Besides hydrolytic enzymes, lytic polysaccharide monooxygenases (LPMOs) and cellobiose dehydrogenases (CDHs) facilitate degradation of plant cell wall polysaccharides by oxidative action (Vaaje-Kolstad et al., 2010; Langston et al., 2011).

Wood decay patterns differ among white-rot fungi (Cantarel et al., 2008). Most of the studied species, including the model white-rot fungus *Phanerochaete chrysosporium*, remove cellulose, hemicellulose and lignin simultaneously (Korripally et al., 2015). On the contrary, the species that degrade lignin prior to polysaccharides are called selective lignin degraders, and include e.g. *Obba rivulosa* and *Gelatoporia* (*Ceriporiopsis*) *subvermispora* (Akhtar et al., 1997; Gupta et al., 2011; Hakala et al., 2004). These species are especially interesting in the biotechnological applications aiming to remove lignin (Hakala et al., 2004; Maijala et al., 2008).

O. rivulosa, a member of the *Gelatoporia* clade, is relatively common in North America (Nakasone, 1981), but sparsely distributed in Africa (Hjortstam and Ryvarden, 1996), Asia (Núñez and Ryvarden, 2001) and Europe (Ryvarden and Gilbertson, 1994), where it has been mostly isolated from coniferous softwood (Hakala et al., 2004). A dikaryotic *O. rivulosa* strain T241i (FBCC949) has been shown to degrade spruce softwood selectively (Hakala et al., 2004). Moreover, the *O. rivulosa* genome encodes a full set of lignocellulose-degrading genes, making it an interesting candidate for plant biomass research (Miettinen et al., 2016). Except for two MnPs and two laccases (Hakala et al., 2005; Hildén et al., 2013), no other lignocellulosic enzymes produced by *O. rivulosa* have been characterized, and therefore its mechanisms for plant cell wall degradation remain largely unknown.

Here we report temporal transcriptome analysis of *O. rivulosa* grown on its natural substrate, spruce wood. We used the genome-sequenced monokaryotic strain 3A-2, derived from the dikaryotic strain T241i, which has been previously studied in terms of selective lignin degradation. The expression of genes encoding putative plant cell wall degrading CAZymes was studied after 2, 4 and 8 weeks of solid state cultivation in order to follow wood depolymerization in more natural like conditions. In addition, central carbon metabolic enzymes and fungal cell acting CAZymes encoding genes were studied to get insights into the nutritional demands during a prolonged cultivation on wood.

2. Materials and methods

2.1. Fungal strain and culture conditions

O. rivulosa monokaryon 3A-2 (FBCC1032) derived from the dikaryotic *O. rivulosa* strain T241i (FBCC949) was obtained from the HAMBI Fungal Biotechnology Culture Collection, University of Helsinki, Helsinki, Finland (fbcc@helsinki.fi). The fungus was maintained on 2% malt extract agar plates (MEA) (2% (w/v) malt extract, 2% (w/v) agar). For pre-cultures, the fungus was cultivated for 7 days at 28 °C in 100 ml liquid low-nitrogen-asparagine-succinate medium (LN-AS), pH 4.5 (Hatakka and Uusi-Rauva, 1983), supplemented with 0.05% glycerol, in 250 ml Erlenmeyer flasks, which were inoculated with five mycelium-covered agar plugs (∅ 7 mm) from MEA plates. After the homogenization (Waring Blender, USA), 4 ml of mycelial suspension was used for the inoculation of spruce wood solid cultures, which consisted of 2 g (dry weight) of Norway spruce (*Picea abies*) wood sticks (approx. 2 × 0.2 × 0.3 cm in size) on 1% (w/v) water agar (Mäkelä et al., 2002). Cultures were incubated stationary at 28 °C in the dark for 2, 4, and 8 weeks. Three replicate control cultures inoculated with 4 ml of LN-AS supplemented with 0.05% glycerol were incubated similarly. After reaching the specific time point, mycelium-colonized wood sticks were flash frozen in liquid nitrogen followed by subsequent RNA extraction.

2.2. RNA extraction, cDNA library preparation and RNA sequencing

Total RNA was extracted from the spruce cultures by using a CsCl gradient ultracentrifugation as described previously (Patyshakuliyeva et al., 2014). Quality and quantity of RNA were determined by using the RNA6000 Nano Assay (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). Purification of mRNA, synthesis of cDNA library, and sequencing (RNA-seq) was performed at the BGI Tech Solutions Co. Ltd. (Hong Kong, China) as described in Patyshakuliyeva et al. (2015). On average, 51 bp sequenced reads were constituted, producing approximately 557 MB raw yields for each sample. RNA-seq data was analyzed and statistically treated as described previously (Patyshakuliyeva et al., 2015). Raw reads were produced by base calling from the original image data. After that, data filtering was performed. Adaptor sequences, reads with unknown bases (N) > 10% and low quality reads (> 50% of the bases with quality values < 5%) were removed. Clean reads were mapped to the genome sequence of *O. rivulosa* 3A-2 (v1.0 annotation, http://genome.jgi.doe.gov/Obbri1, (Miettinen et al., 2016)) using BWA/Bowtie (Langmead et al., 2009; Li and Durbin, 2010). On average, 82% total mapped read to the gene was achieved. The expression level was calculated as Fragments Per Kilobase of exon model per Million fragments mapped (FPKM) by using RSEM tool (Li and Dewey, 2011). Genes with FPKM value < 20 under all conditions were considered as not expressed and filtered out of the analysis, and genes showing FPKM value ≥ 20 were considered as significantly expressed. Genes with FPKM value from 20 to 100 were considered as lowly, 100 to 300 as moderately and over 300 as highly expressed (approximately top 10% of the genes). Differential expression was identified by Student's T-test. A cut-off of fold change of > 1.5 and P-value of < 0.05 were used to identify differentially expressed genes between the time points. Genome-wide principal component analysis (PCA) of the gene expression on duplicate samples of the three time points was generated using FactoMineR package from Rcomander v.2.1-7 program in R statistical language and environment 3.1.2. (Lê et al., 2008). The RNA-seq data have been submitted to Gene Expression Omnibus (GEO) (Edgar et al., 2001) with GEO ID: GSE99871.

2.3. Validation of RNA-seq expression patterns by qRT-PCR

Smart RACE cDNA Amplification Kit (Clontech) was used for the cDNA synthesis according to the manufacturer's instructions. 1 μg of RNA originating from two replicate cultures of *O. rivulosa* that were used in RNA-seq was converted to cDNA in 20 μL reaction with Smart RACE cDNA Amplification Kit (Clontech) and SuperScript III reverse transcriptase (Invitrogen) according to the instructions of the manufacturers.

The relative amounts of nine selected gene transcripts were determined by qRT-PCR analysis to validate the RNA-seq expression

patterns. Gene-specific primers spanning exon-exon junction (Supplementary Table 1) were designed according to the genome of *O. rivulosa* 3A-2 (http://genome.jgi.doe.gov/Obbri1, Miettinen et al., 2016) with PerlPrimer software (Marshall, 2004).

The amplification efficiency (E) of the primers was calculated from the slope of standard curve made with template cDNA serial dilutions using the formula: $E = [10^{(-1/\text{slope})} - 1] \times 100$. The E-values of the primer pairs varied from 94% to 102%, whereas, the R^2 values, ranged from 0.993 to 0.999 (Supplementary Table 1).

Three technical replicate qRT-PCR reactions were conducted for each sample and primer pair using CFX96 Real-Time System C1000 Touch Thermal Cycler (Bio-Rad, USA). The 20 µL reactions comprised of 30 ng cDNA template, 0,4 µM forward and reverse primer, 1 X DyNAmo HS SYBR Green qPCR master mix (Thermo Scientific), and H_2O to the final volume of 20 µL. Cycling protocol was: initial denaturation at 95 °C followed by 35 cycles of (1) denaturation at 94 °C for 10 s, (2) annealing at 56 °C for 20 s, and (3) extension at 72 °C for 30 s. Fluorescence data acquisition was done during the extension step. To confirm the specificity of the qRT-PCR primers, melting curve was generated and inspected for the presence of a single peak. Relative expression levels were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) for the *gapdh*-normalized cycle threshold (Ct) values and the results are reported as relative fold changes.

3. Results

The monokaryotic *O. rivulosa* 3A-2 strain grown on solid spruce wood was subjected to the time-scale transcriptomics study to compare the gene expression at different growth stages of the fungus. PCA analysis showed that the duplicate RNA samples used for RNA-seq were highly reproducible (Supplementary Fig. 1). In addition, the expression patterns obtained by RNA-seq analysis corresponded well to the qRT-PCR results of the nine selected putative CAZyme-encoding genes (Fig. 1). Similar to other Polyporales species, the genome of *O. rivulosa* contains a full repertoire of putative CAZyme-encoding genes targeted to plant cell wall degradation (Miettinen et al., 2016). In total, 259 different putative CAZyme-encoding genes were significantly expressed in *O. rivulosa* cultures, and of these 110 were predicted to encode plant cell wall degrading enzymes (PCWDEs) (Fig. 2A) from 35 different CAZy families (Supplementary Table 2). Transcripts encoding putative GHs were the most abundant ones with 138 detected transcripts (53% of total PCWDE CAZys). The CAZymes-encoding transcripts from AAs, GTs, CEs, and PLs represented 15%, 30%, 7%, and 2.5% of the total PCWDE CAZy transcripts detected, respectively. Of these, 20, 31, and 16 CAZy genes are putatively targeted towards cellulose, hemicellulose and lignin, respectively (Fig. 2A). 16 genes encoding putatively H_2O_2-supplying enzymes were detected, while 6 and 5 genes encoding pectin and starch depolymerizing enzymes were expressed, respectively. Diverse activities included 16 CAZy transcripts, which can have activity towards multiple substrates. Highly expressed PCWDE CAZyme encoding transcripts belonged to 20 different CAZy families with varying gene numbers (Fig. 2B). All genes from cellulose acting GH6, GH12 and AA8-A3_1, xyloglucan acting GH73 and pectin acting GH53 and GH88 families showed high expression (Fig. 2B).

As a typical white-rot fungal species, *O. rivulosa* harbors multiple genes predicted to be involved in lignin degradation in its genome. Of these, two putative MnP (Protein IDs 806545 and 835392) encoding genes were the highest expressed PCWDE CAZy transcripts during the cultivation. Interestingly, they showed unusually high expression levels throughout the cultivation peaking from 20 000 to 22 000 FPKM after 4-week cultivation (Table 1). This may suggest an important role for the corresponding enzymes in lignin degradation during the growth of *O. rivulosa* monokaryon on spruce. Three putative AA1_1 laccases were also detected on spruce, but none of those was highly expressed (Supplementary Table 3A). In addition to the laccases, *O. rivulosa* multicopper oxidase-encoding transcripts included one AA1_2 ferroxidase (FET), which compared to the laccases was moderately expressed. Production of hydrogen peroxide is essential for the lignin degradation since it is a prerequisite for peroxidase activity. In accordance with that, 10 transcripts predicted to encode family AA3_2 and AA3_3 glucose methanol choline (GMC) oxidoreductases were detected. Although most of the transcripts encoding the putative GMCs showed low expression, interestingly, one of the transcripts encoding an AA3_3 alcohol oxidase (AOX; 790443) was highly expressed in all time points, and was the most abundant transcript among lignin degradation-related transcripts after two AA2 MnPs. These findings confirm the major importance of peroxidases and hydrogen peroxide-producing machinery for the lignocellulose conversion in *O. rivulosa*.

Of the transcripts predicted to encode cellulolytic activities, β-1,4-endoglucanases (EGLs), cellobiohydrolases (CBHs), β-1,4-glucosidases (BGLs) and LPMOs were the most abundant ones among all the growth stages of *O. rivulosa* on wood (Supplementary Fig. 2, Supplementary Table 3A). In total, 9 transcripts encoding putative EGLs from GH5, 12, 45 and 131 were detected. Six of those were highly expressed after 2 weeks with a 98- to 17- fold downregulation at the later stages (Supplementary Table 3A). The *O. rivulosa* genome possesses one GH6 cellobiohydrolase II (CBHII) and two GH7 CBHIs, of which the CBHII (Protein ID 476379) as well as one CBHI (Protein ID 731121) were highly expressed after 2 weeks. Interestingly, their expression decreased to moderate and low levels after 4 and 8 weeks of cultivation, respectively. A third important hydrolytic cellulose-acting enzymes are encoded by the enzyme families GH1 and GH3. Although seven BGL encoding genes were present in the transcriptome, only one GH3 BGL (Protein ID 14692) was highly expressed after 2-week cultivation. Genes encoding seven putative LPMOs oxidatively cleaving plant cell wall polysaccharides were also expressed (Supplementary Table 3A). Of these, six were found to be highly expressed, most of them after 2 weeks of cultivation. Interestingly, one LPMO (Protein ID 794851) encoding gene was highly expressed throughout the cultivation.

The most abundant transcript predicted to act on hemicellulose was a GH5 mannanase (MAN; Protein ID 641261), which was the third most abundant transcript in general (Table 1). Overall, three transcripts predicted to encode GH5 MANs were detected. Among four detected GH27 α-galactosidases (AGLs), only one was highly expressed (Protein ID 849432). Six genes encoding GH10 endoxylanases, 4 of which were highly expressed at the early cultivation stage (Protein IDs 838746, 851185, 762583 and 799009), were detected in the cultures. In addition, genes encoding one putative α-glucuronidase (AGU; Protein ID 726547) and one putative xyloglucanase (XG-EG; Protein ID 808997) showed high expression levels. Interestingly, almost all predicted hemicellulose degrading enzyme encoding genes showed higher abundances in early stages of wood decay with subsequent decrease. Only one GH2 mannosidase (MND; Protein ID 753990) was expressed at a constant, moderate level at all three time points. High expression was not detected for any of the putative hemicellulases encoding genes after 4 or 8 weeks of cultivation.

In addition to the hemicellulose specific GHs, differential abundances of transcripts predicted to encode one hemicellulose acting glucuronoyl esterase (GE; Protein ID 762191) and three multiple substrates acting acetyl esterases (AE; Protein IDs 749512, 724015 and 816606) from carbohydrate esterase (CE) families 15 and 16, respectively, were detected. Similar to hemicellulose degrading GH families, their expression trend showed the highest transcript abundances in early cultivation stage.

Overall, the expression of the PCWDE CAZy genes was highest after 2-week growth of *O. rivulosa* and reduced markedly over time (Supplementary Table 3A, Supplementary Fig. 2). The only exceptions were MnPs, LCCs, AAOs and a single copy of PGA and GAL. When the sum of the transcript levels per putative CAZy enzyme activity was compared during the cultivation, MnPs were the most highly expressed, followed by LPMOs, XLNs, CBHs and MANs, respectively (Supplementary Fig. 2).

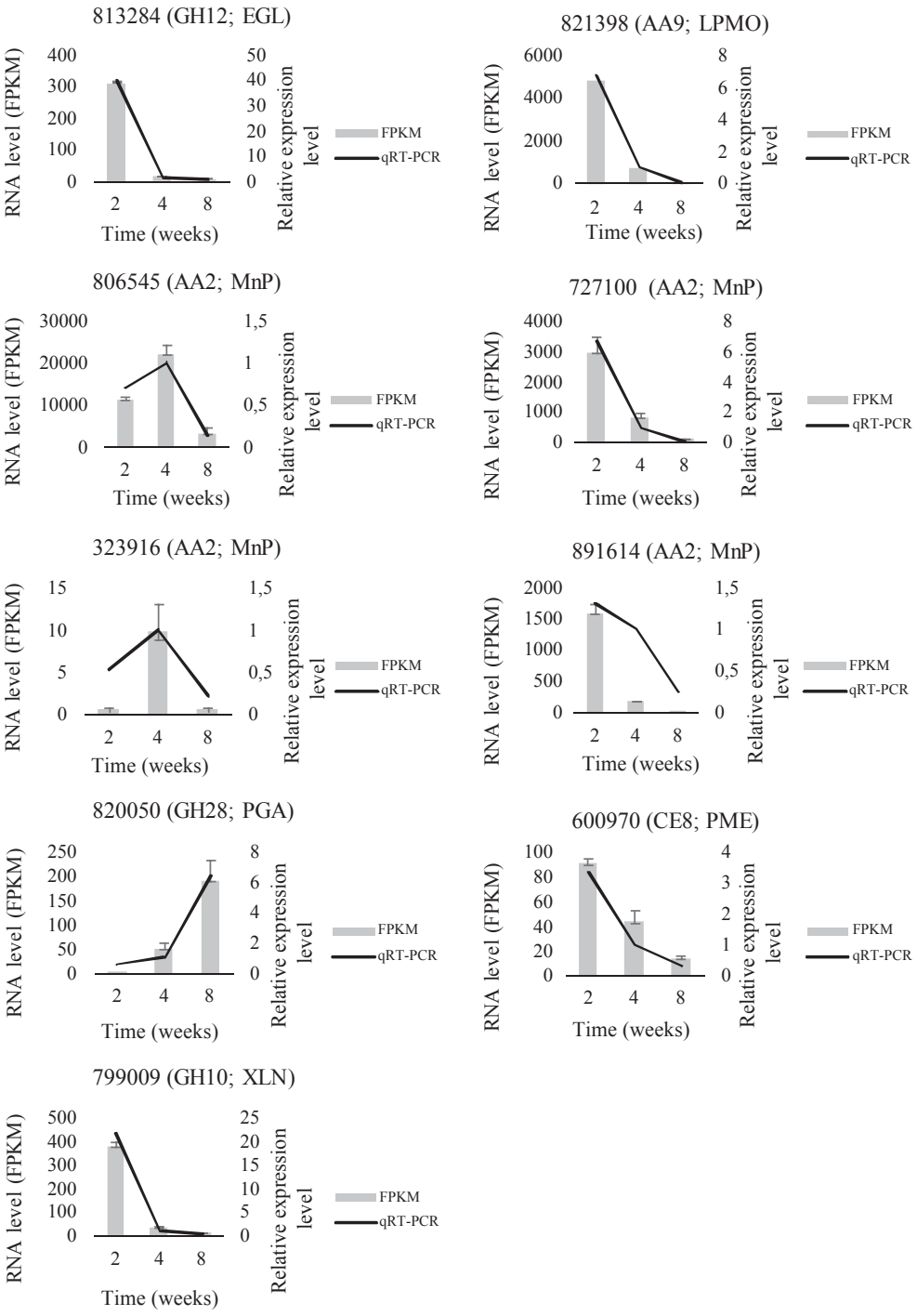

Fig. 1. Validation of RNA-seq analysis by qRT-PCR of nine selected genes involved in plant cell wall degradation in *O. rivulosa*. Columns represent RNA level (FPKM), lines represent qRT-PCR values (relative unit). Error bars represent standard deviation of two biological replicates and three replicate qRT-PCR reactions. Enzyme abbreviations are presented in Supplementary Table 2.

Altogether 22% (13 out of 60) of predicted fungal cell wall encoding CAZymes were highly expressed in all three time points (Supplementary Table 3B). These genes showed an interesting trend of either being highly expressed throughout the cultivation or showing high level expression at the early stage with a decreasing trend after 4-week cultivation, followed by an upregulation at the last time point after 8 weeks (Supplementary Table 3B). This could suggest possible recycling of fungal cell wall polysaccharides by *O. rivulosa*, such as α-1,3-, β-1,3- and β-1,6-glucans, which can be hydrolyzed to glucose and reutilized by the fungus. The overall highest transcript abundancy was detected for one gene encoding a putative GH131 β-glucanase (Protein ID 812963), acting on β-(1,3)-/β-(1,6)- and β-(1,4)-linked glucan substrates, after 2-week cultivation. Other highly expressed genes in the early time point were two GH16 β-1,3(4)-endoglucanases, a GH18 chitinase and GH55 β-1,3-endoglucanase. Constantly highly expressed transcripts included a CE4 chitin deacetylase, a GH16 β-1,3(4)-endoglucanase, two β-1,3-endoglucanases from GH16 and GH128, a GH16 licheninase and a GH18 chitinase.

Despite the downregulated expression of the genes encoding CAZymes targeted for carbon acquisition from plant biomass, *O. rivulosa* showed active mycelial growth throughout the cultivation (Fig. 3). This was also confirmed by the expression of the carbon metabolic genes showing that all central carbon metabolic pathways were active in all studied time points. This suggests that *O. rivulosa* was not under carbon starvation during the cultivation (Supplementary Table 3C, Supplementary Fig. 3). D-glucose, D-mannose and D-xylose are the

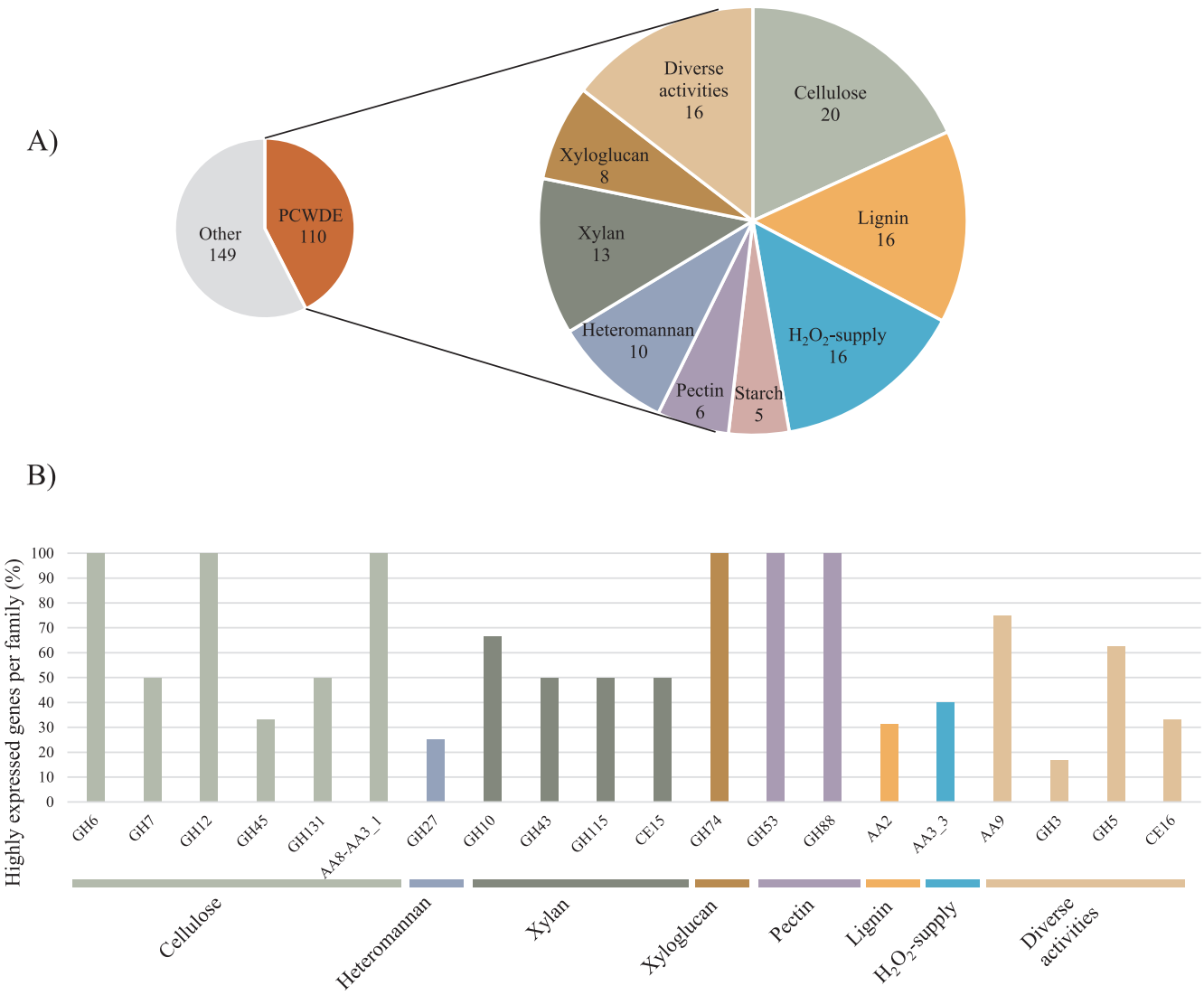

Fig. 2. (A) Functional distribution of detected putative CAZyme encoding genes identified in *O. rivulosa* spruce cultures. (B) Highly expressed (> 300 FKPM) putative CAZyme encoding genes per CAZy family as a percentage of total gene number per family. Substrates, the corresponding enzymes putatively act on, are indicated. PCWDE, plant cell wall degrading enzymes.

major monosaccharides originating from spruce wood polysaccharides, while smaller amounts of D-galactose, L-arabinose and L-rhamnose are also present (Rytioja et al., 2017). Hexose monomers can be converted through glycolysis, which is connected to pentose phosphate pathway (PPP). Among the glycolysis genes, *pki1*, encoding pyruvate kinase, catalyzing the last step of the glycolysis, as well as genes encoding glucose-6-phosphate isomerase (*pgi1*), fructose-2,6-bisphosphatase (*pfk2*), fructose-bisphosphate aldolase (*fba1*), glyceraldehyde-3-phosphate dehydrogenase (*gpd1*), and enolase (*eno1*), showed high expression (Supplementary Fig. 3A). While *pgi1*, *pfk2* and *fba1* showed decreasing expression during the cultivation, *gpd1* and *eno1* were upregulated. Isocitrate lyase (*icl1*), malate dehydrogenase (*mdh1*) and related oxaloacetase (*oxa1*) encoding genes involved in the tricarboxylic acid (TCA) cycle were highly expressed (Supplementary Fig. 3A). A gene encoding 6-phosphogluconate dehydrogenase (*gnd1*) as well as one of the transaldolases encoding genes (*tal2*) of the PPP showed constant high level expression throughout the cultivation (Supplementary Fig. 3B). Pentoses D-xylose and L-arabinose originating from hemicelluloses and pectin are catabolized through the pentose catabolic pathway (PCP). High level expression was detected for two out of the three PCP genes identified in *O. rivulosa*, i.e. L-arabinose reductase (*lar1*) and xylitol dehydrogenase (*xdh1*) (Supplementary Fig. 3B). However, all three PCP genes were downregulated after 2-week growth. It should be noted that we were not able to identify (based on similarity to known ascomycete genes) some of the carbon metabolic genes in *O. rivulosa*, including half of the genes encoding PCP enzymes (Supplementary Fig. 3). The pathways for catabolism of pectin-derived L-rhamnose and D-galacturonic acid, as well as the Leloir pathway for D-galactose present in hemicelluloses and pectin were also active (Supplementary Fig. 3C).

4. Discussion

In this work, we studied the transcriptomic response of the white-rot fungus *O. rivulosa* during a prolonged cultivation period of 8 weeks on solid spruce to evaluate changes in gene expression during the lengthy process of fungal wood colonization. The genome-sequenced monokaryotic *O. rivulosa* strain 3A-2 (Miettinen et al., 2016) was used, and the focus was on the analysis of the genes encoding plant cell wall polymers degrading CAZymes that are responsible for carbon acquisition from plant biomass. Also, carbon metabolic genes and fungal cell wall degrading enzymes were evaluated to get a collective overview of ongoing metabolic processes.

Our results show that *O. rivulosa* highly expresses the genes

Table 1
The highest expressed CAZyme encoding genes across 8-week cultivation of *O. rivulosa* on spruce. Only genes showing FPKM values higher than 1000 at any time point are presented.

Protein ID	CAZy family	Functional annotation	RNA level (FPKM)		
			2 weeks	4 weeks	8 weeks
806545	AA2	MnP	11194	21804	3071
835392	AA2	MnP	9970	19896	2638
641261	GH5_7	MAN	7664	257	116
838746	GH10	XLN	6589	176	28
821398	AA9	LPMO	4789	729	28
476379	CBM1-GH6	CBHII	4781	165	56
790443	AA3_3	AOX	4312	1889	551
731121	GH7-CBM1	CBHI	4121	287	50
727100	AA2	MnP	2938	803	85
833133	AA9-CBM1	LPMO	2878	439	70
788967	CBM1-GH5_5	EGL	2032	188	103
851185	GH10	XLN	1954	83	5
726082	GH12	EGL	1585	121	38
891614	AA2	MnP*	1565	174	2
749512	CE16	AE	1428	145	29
781628	AA9-CBM1	LPMO	1290	268	102
789780	CBM1-GH5_5	EGL	1268	152	73
812963	GH131	EGL	1211	79	24
719765	GH5_7	MAN	1201	43	29
724015	CE16	AE	1125	129	35

* Hakala et al. (2006).

encoding a complete repertoire of enzymes for degradation of cellulose, hemicellulose and lignin on spruce, similarly to other white rot species including *Dichomitus squalens* (Rytioja et al., 2017) and *Phlebia radiata* (Kuuskeri et al., 2016). Interestingly, almost all of the detected plant cell wall acting CAZy genes were highly expressed after 2-week growth of *O. rivulosa*, while most of them were strongly downregulated in the later time points. Generally regarded as key enzymes of white rot fungal ligninolytic system, class II heme peroxidases are abundantly represented in the genome of *O. rivulosa*, including 11 MnPs, and one LiP from family AA2 (Miettinen et al., 2016). Among nine putative MnP encoding transcripts detected in spruce cultivations, five genes were highly expressed of which two (Protein IDs 806545 and 835392) exhibited unusually high FPKM levels throughout the cultivation and had the highest transcript abundances at 4-week time point. MnPs have often been shown to be constantly produced by white-rot fungi throughout the solid state cultivation on wood (Aguiar et al., 2006; Galliano et al., 1991; Hakala et al., 2005), which is in line with the high expression of the two putative MnP transcripts of *O. rivulosa*.

Expression levels of the LiP encoding gene were negligible (FPKM < 20) suggesting only minor input in lignin degradation by *O. rivulosa*. This is in accordance with the results from *G. subvermispora*, a close relative of *O. rivulosa*, where a putative LiP was not upregulated in aspen cultures compared to glucose medium (Fernandez-Fueyo et al., 2012). On the contrary, the non-selective white-rot fungi, such as *P. chrysosporium* (Vanden Wymelenberg et al., 2010), *Phanerochaete carnosa* (MacDonald et al., 2011) and *P. radiata* (Kuuskeri et al., 2016), have shown significant upregulation of several LiP encoding genes on wood substrates, implying differences in lignin degradation approaches between the white-rot fungal species.

Three out of nine putative AA1_1 laccase genes of *O. rivulosa* were expressed on spruce, but none of those was highly expressed. This is in contrast with *G. subvermispora* (Fernandez-Fueyo et al., 2012) and *D. squalens* (Rytioja et al., 2017) laccases, which were significantly upregulated in the agitated liquid cultures supplemented with milled aspen wood and spruce sawdust, respectively. However, our finding was consistent with that from the solid spruce cultures of the white-rot fungus *P. radiata*, showing low expression of laccase-encoding genes after 4-weeks of cultivation (Kuuskeri et al., 2016). This may indicate that in the shaken liquid cultures, laccases defend the fungal mycelium against oxidative stress (Jaszek et al., 2006; Joo et al., 2008), whereas in the more natural like solid state cultivations, their role in lignin degradation is controversial. Nevertheless, acidic laccase isoforms have been purified from the early phase of the solid spruce chip cultures of *O. rivulosa* despite of the minor laccase activity detected from the cultivations (Hakala et al., 2005). This could indicate that laccases may have a role in initial wood colonization.

The dikaryotic *O. rivulosa* strain T241i has been shown to selectively degrade spruce by decomposing lignin prior to cellulose (Hakala et al., 2004). However, the results of our study do not indicate selective lignin degradation by the monokaryotic *O. rivulosa* 3A-2, as genes encoding enzymes targeted towards all plant biomass polymers were simultaneously highly expressed. A high level of diversity within the white rot fungal species has often been reported. These include discrepancies regarding enzyme production profile and lignin degrading ability between *Pleurotus osteratus* monokaryon and its parental dikaryon. In solid state fermentation, the monokaryon showed higher lignin-modifying

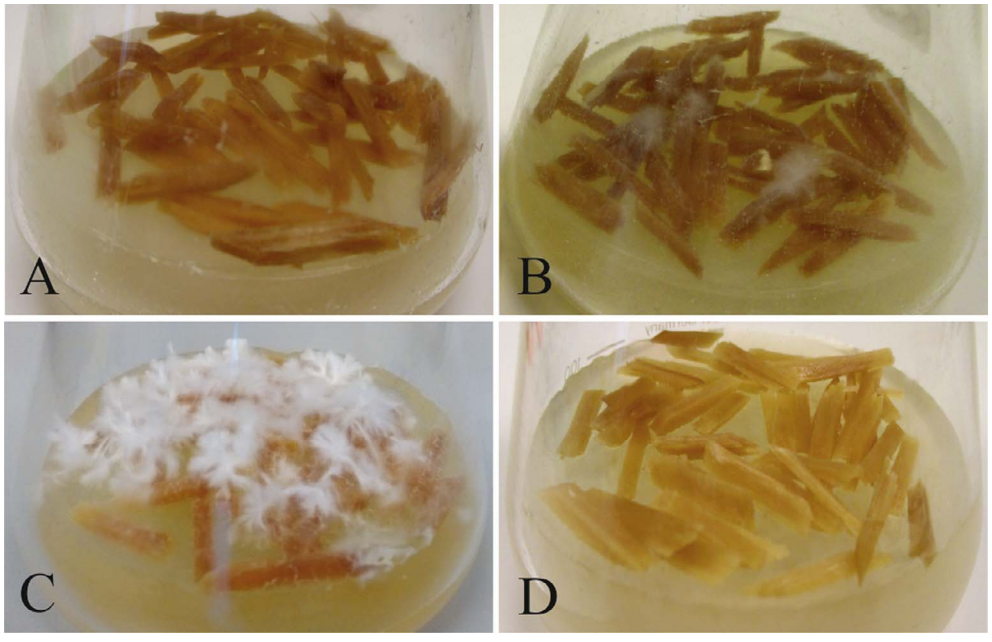

Fig. 3. *O. rivulosa* monokaryon 3A-2 grown on solid spruce wood sticks for (A) 2 weeks, (B) 4 weeks and (C) 8 weeks. (D) Non-inoculated control cultivation.

enzyme activities, but a lower rate of lignin degradation compared to the dikaryon (Eichlerová et al., 2000). Highly variable lignocellulose acting enzyme profiles have also been detected between the mono- and dikaryotic strains of *D. squalens* (Casado-López et al., 2017). In addition, higher levels of ligninolytic enzyme activities have been produced by monokaryotic strains of the white-rot fungi *Pycnopours cinnabarinus* (Herpoël et al., 2000), *Pycnoporus sanguineus* (Lomascolo et al., 2002), *P. ostreatus* (Eichlerová et al., 2002) and *Trametes hirsuta* (Li et al., 2012) compared to the parental dikaryon.

Selective lignin degradation seems also to be temporally regulated, as the selectivity is usually limited to early stages of decay (Adaskaveg et al., 1995; Ferraz et al., 2000). It may be possible that the 2-week time point was too late to detect the initial lignin degradation selectivity at the transcript level in the *O. rivulosa* 3A-2 cultures, although its parental dikaryon T241i has maintained the selectivity during prolonged cultivation (Hakala et al., 2004). Selectivity of white-rot wood degradation is also dependent on the physical and chemical parameters, such as temperature, and oxygen and moisture content, in wood (Adaskaveg et al., 1995; Blanchette, 1995). *O. rivulosa* T241i has been shown to degrade lignin selectively when grown on spruce wood blocks at 25 °C (Hakala et al., 2004), thus differing slightly from the conditions used in this study for *O. rivulosa* 3A-2.

In addition to plant polymers degrading enzymes of *O. rivulosa* assessed in this study, we also evaluated enzymes involved in the major carbon metabolic pathways. Carbon catabolic genes were expressed throughout the 8-week cultivation demonstrating good metabolic activity of the fungus during lengthy wood colonization and conversion in laboratory conditions. Overall, higher expression was detected for genes encoding enzymes involved in glycolysis and PPP than for those involved in PCP. A similar trend has been reported from the compost grown litter decomposing *Agaricus bisporus* (Patyshakuliyeva et al., 2015), possibly suggesting a preferred use of hexoses over pentoses. Among TCA and glyoxalate cycle related genes, malate dehydrogenase (*mdh1*) and oxaloacetase (*oxa1*) showed the highest transcript levels during growth of *O. rivulosa* on spruce. Oxalate synthesis has been suggested to be coupled with energy production in brown-rot fungi *Fomitopsis palustris* (Munir et al., 2001) and *Postia placenta* (Martinez et al., 2009) by Mdh1, which generates energy by oxidizing malate to oxaloacetate, which is then converted to oxalate by Oxa1. In addition, a number of roles for oxalate with respect to lignocellulose degradation has been proposed, including acidification of fungal extracellular environment to the levels that are usually needed for the activity of lignin acting enzymes (Mäkelä et al., 2010). *O. rivulosa* has been shown to produce oxalate during growth on spruce wood chips (Hakala et al., 2005), which is in line with the constant high expression of *oxa1* in *O. rivulosa* observed in our study.

Fungal cell wall acting enzymes comprising mostly chitinases and various β-glucanases are important for cell wall remodeling during active hyphal growth, as well as aging-related cell wall recycling (Gruber and Seidl-Seiboth, 2012). The most numerous representatives of the genes encoding putative fungal cell wall acting enzymes expressed by *O. rivulosa* were family GH16 members that are involved in chitin-β-1,3-glucan formation, suggesting a role in the processing of the fungal cell wall polysaccharides (Klis et al., 2007). Two out of 11 putative GH18 chitinases encoding genes of *O. rivulosa* showed constant high-level expression suggesting continuous recycling of chitin during the cultivation. In ectomycorrhizal fungus *Laccaria bicolor* GH18 chitinase genes are also found to be upregulated in free-living mycelium implying possible degradation of exogenous fungal cell wall (Veneault-Fourrey et al., 2014).

Our results suggest that during the growth of *O. rivulosa* monokaryon 3A-2 on its natural substrate, spruce wood, the highest expression of genes involved in plant cell wall degradation occurs at early stages of wood colonization. The simultaneous expression of genes targeted towards all lignocellulosic polymers suggests that *O. rivulosa* 3A-2 does not selectively remove spruce wood lignin. Thus, these results indicate high variation within mono- and dikaryotic strains of white-rot fungal species towards lignocellulose degradation.

Acknowledgements

MM was supported by the Academy of Finland research grant no: 297847 and the European Commission, Marie Curie ITN network Subicat FP7 Grant agreement no: 607044, and OM by Marie Curie fellowships PIOF-GA-2011-302349. MVA-P was supported by a grant of the Dutch Technology Foundation STW, Applied Science division of NWO, and the Technology Program of the Ministry of Economic Affairs 016.130.609 to RPdV.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2017.07.004.

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