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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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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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(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