Fungal Genetics and Biology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Fungal Genetics and Biology

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

Transcriptional analysis of selected cellulose-acting enzymes encoding genes of the white-rot fungus *Dichomitus squalens* on spruce wood and microcrystalline cellulose

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
White-rot fungus
Dichomitus squalens
Cellobiohydrolase
Cellobiose dehydrogenase
Lytic polysaccharide monooxygenase

ABSTRACT

The recent discovery of oxidative cellulose degradation enhancing enzymes has considerably changed the traditional concept of hydrolytic cellulose degradation. The relative expression levels of ten cellulose-acting enzyme encoding genes of the white-rot fungus *Dichomitus squalens* were studied on solid-state spruce wood and in microcrystalline Avicel cellulose cultures. From the cellobiohydrolase encoding genes, *cel7c* was detected at the highest level and showed constitutive expression whereas variable transcript levels were detected for *cel7a*, *cel7b* and *cel6* in the course of four-week spruce cultivation. The cellulolytic enzyme activities detected in the liquid cultures were consistent with the transcript levels. Interestingly, the selected lytic polysaccharide monooxygenase (LPMO) encoding genes were expressed in both cultures, but showed different transcription patterns on wood compared to those in submerged microcrystalline cellulose cultures. On spruce wood, higher transcript levels were detected for the *lpmos* carrying cellulose binding module (CBM) than for the *lpmos* without CBMs. In both cultures, the expression levels of the *lpmo* genes were generally higher than the levels of cellobiose dehydrogenase (CDH) encoding genes. Based on the results of this work, the oxidative cellulose cleaving enzymes of *D. squalens* have essential role in cellulose degrading machinery of the fungus.

1. Introduction

Plant biomass is an abundant renewable carbon source with a huge biotechnological potential as a raw material in several industries. For the efficient and feasible use of lignocellulosic plant biomass, depolymerisation of the plant cell wall polymeric components, including the polysaccharides cellulose and hemicelluloses, and the aromatic lignin, is needed.

Fungi are the most effective plant cell wall degrading organisms producing diverse set of synergistically acting extracellular enzymes (Eriksson et al., 1990; Lundell et al., 2010; Hatakka and Hammel, 2011). The main catalytic activities of lignocellulose degrading fungi for cellulose decomposition are various hydrolytic enzymes such as endoglucanases (EG, EC 3.2.1.4) and exoglucanases (cellobiohydrolase I and II; CBHI and CBHII; EC 3.2.1.176 and EC 3.2.1.91, respectively), and β-glucosidases (BGL, EC 3.2.1.21,). They belong to different glycoside hydrolase families (GH) classified in the Carbohydrate-Active enZyme database (CAZy, www.cazy.org) (Cantarel et al., 2009). In addition, oxidative enzymes of Auxiliary Activities (AA) group (Levasseur et al., 2013) such as cellobiose dehydrogenase (CDH, AA3, AA8; EC 1.1.99.18) and lytic polysaccharide monooxygenases (LPMOs, AA9) have been shown to enhance the degradation of cellulose in combination with cellulases (Harris et al., 2010; Quinlan et al., 2011). Furthermore, basidiomycetous white-rot fungi produce lignin-modifying oxidoreductases, such as class II haem-peroxidases and laccases, endowing them the unique ability to depolymerize lignin, which is the most recalcitrant part of plant cell wall (Lundell et al., 2010).

So far, most of the studies on cellulose degrading fungal enzymes have been concentrated on ascomycetous fungi. The accumulating genome data, however, reveals that also basidiomycetous white-rot fungi have a complete set of plant polysaccharide decomposing enzymes (Floudas et al., 2012). Since the focus of white-rot fungal research has mostly been on the oxidoreductive lignin-modifying enzymes, basidiomycetous carbohydrate active enzymes (CAZymes) have been studied to a lesser extent and thus analyses both on transcriptional and biochemical level are still scarce (Baldrian and Valášková, 2008). Recently, ascomycetous LPMOs have been reported to enhance cellulose degradation by opening of the cellulose chain oxidatively thus suggesting an important role of these enzymes together with classical cellulases (Quinlan et al., 2011). Moreover, the action of LPMO is strongly potentiated when combined with CDH (Langston et al., 2011;

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http://dx.doi.org/10.1016/j.fgb.2013.12.008

Phillips et al., 2011; Beeson et al., 2012; Li et al., 2012; Bey et al., 2013). The finding of LPMOs may in fact represent a revolution in enzymatic biomass processing (Horn et al., 2012). This has urged to study the role of LPMO–CDH couples also in wood rotting basidiomycetous fungi to fully understand their roles in cellulose biodegradation.

Dichomitus squalens is a white-rot basidiomycete capable of efficient cellulose and lignin degradation on spruce (Hakala et al., 2004; Fackler et al., 2006). The genome of *D. squalens* encodes four CBHs (one from GH6 and three from GH7 family), three putative endoglucanases (GH5) and six putative β-glucosidases (GH3) (Floudas et al., 2012), which hydrolyse cellulose and cleave the β-(1,4)-linkages of D-glucose chain. In addition, *D. squalens* harbours one putative CDH and 15 LPMO encoding genes (Floudas et al., 2012) that are likely to participate in the oxidative cleavage of cellulose. To complete the degradation, *D. squalens* has e.g. genes encoding 9 manganese peroxidases (MnPs) and 11 laccases, as well as several hemicellulose-acting enzymes for lignin and hemicellulose modification or degradation, respectively. Therefore, *D. squalens* possessing wide variety of the extracellular lignocellulose-modifying enzymes encoding genes and producing the active enzymes is an excellent model fungus for detailed studies on lignocellulose degradation.

This study presents the relative expression of CBH, CDH and selected putative LPMO encoding genes of *D. squalens* when the fungus grows on its natural substrate, i.e. solid spruce (*Picea abies*) wood, or in liquid cultures supplemented with microcrystalline cellulose. The results showed substrate- and time-dependent variation in the expression of the studied genes. However, all selected genes were transcribed in both cultures thus indicating simultaneous production of cellulose hydrolysing and oxidising enzymes.

2. Materials and methods

2.1. Fungal cultivations

D. squalens FBCC312 was obtained from the Fungal Biotechnology Culture Collection (FBCC), Department of Food and Environmental Sciences, University of Helsinki (email: fbcc@helsinki.fi), and the fungus was maintained on 2% malt agar plates (2% (wt/vol) malt extract (Biokar, France), 2% (wt/vol) agar agar (Biokar, France). For the fungal inoculum, *D. squalens* was cultivated in 250 ml Erlenmeyer flasks containing 75 ml liquid 2% (wt/vol) malt extract medium, which was inoculated with three mycelium-covered plugs (Ø 7 mm) from malt agar plates and incubated stationarily for 7 d at 28 °C in dark.

Solid-state spruce wood cultures contained 2 g (dry weight) of Norway spruce (*Picea abies*) wood sticks on top of the 1% (wt/vol) water agar. Moisture content of the spruce wood cultures was adjusted to 60% with sterile water. The cultures were inoculated with 2 ml of homogenised fungal mycelium (Mäkelä et al., 2002) from the malt extract cultures and incubated at 28 °C in dark for 28 d.

Microcrystalline cellulose (Avicel) cultures of *D. squalens* were performed in 250 ml Erlenmeyer flasks containing 100 ml liquid medium (pH 6.0), which consisted of 2.5 g/l meat peptone (Lab M Limited, UK), 1 g/l yeast extract (Lab M Limited, UK), 1 g/l potassium dihydrogen phosphate (Sigma–Aldrich, Japan), 0.5 g/l magnesium sulphate (Merck, Germany) and 1% (wt/vol) Avicel® PH-101 cellulose (Fluka, Ireland). The Avicel cultures were inoculated with 4 ml of homogenised mycelial suspension and incubated under agitation (120 rpm) at 28 °C in dark for 28 d.

2.2. Enzyme activity and protein concentration measurements

Extracellular enzyme activities and protein concentration were determined from the Avicel culture liquids from three replicate cultivations. All the measurements were performed in 96 well plates using Tecan Infinite M200 plate reader (Tecan, Austria).

Cellobiohydrolase (CBH), endoglucanase, β-glucosidase and xylanase activities were determined in 50 mM Na-citrate buffer, pH 5.0, at 45 °C. CBH activity was measured by using 1.6 mM 4-methylumbelliferyl-β-D-lactoside (MULac; Biokemis, Russia) as a substrate in the 100 µl reactions (van Tilbeurgh et al., 1982, 1988). 0.01 M glucose (Sigma–Aldrich, France) or 0.01 M glucose and 0.5 mM cellobiose (Fluka, Slovakia) were used as inhibiting sugars. Reaction was stopped with 1 M sodium carbonate (Sigma–Aldrich, USA) after 10 min and the absorbance was measured at 370 nm. 7-Hydroxy-4-methylcoumarin (MU, Aldrich, Switzerland) was used as a standard.

Endo-β-1,4-glucanase and xylanase activities were measured by using 1% (wt/vol) hydroxyethyl cellulose (HEC, Sigma, USA) (Bailey and Nevalainen, 1981) and 1% (wt/vol) birch or beech xylan (Sigma, Germany) (Bailey et al., 1992) as substrates and with glucose and xylose (Sigma–Aldrich, Germany) as standards, respectively. The reactions of 10 and 5 min, for endo-β-1,4-glucanase and xylanase, respectively, were stopped with 100 µl of dinitrosalisylic acid (DNS) reagent, after which the samples were boiled for 5 min. After cooling, the absorbance was measured at 540 nm.

Activity for β-glucosidase was determined by using 1 mM 4-nitrophenyl β-D-glucopyranoside (Applied Chemical Laboratories, USA) (Bailey and Nevalainen, 1981) as a substrate. The 10 min reaction was stopped with 1 M sodium carbonate and the absorbance was measured at 400 nm using *p*-nitrophenol as a standard.

Laccase activity was measured at 476 nm by detecting the oxidation of 2,6-dimethoxyphenol (Aldrich, Germany) at pH 3.0 in 50 mM sodium malonate at 25 °C (Slomczynski et al., 1995). Manganese peroxidase (MnP) activity was determined by following the formation of Mn^{3+}-malonate complex at 270 nm in 50 mM sodium malonate buffer, pH 4.5, at 25 °C (Wariishi et al., 1992).

Protein concentration was measured with Bradford Reagent according to the manufacturer's instructions (Sigma–Aldrich, USA).

2.3. RNA extraction and cDNA synthesis

The fungal-colonised spruce sticks and the fungal mycelia from the submerged Avicel cultures were milled with grinder (IKA, Germany) and ground in mortar, respectively, under liquid N_2. Total RNA was extracted by N-cetyl-N,N,N-trimethylammonium bromide (CTAB, Sigma, Germany)-based method described by Chang et al. (1993). Concentration of DNaseI (RNase free, Fermentas)-treated RNA was determined spectrophotometrically at 260 nm using NanoDrop ND-1000 (NanoDrop Technologies Inc., USA).

Prior to cDNA synthesis, the RNA was used as a template in real-time quantitative PCR (RT-qPCR) with *D. squalens* glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) primers (see 2.5 RT-qPCR) to confirm that the RNA samples contained no genomic DNA contamination. Smart RACE cDNA Amplification Kit (Clontech) was used for cDNA synthesis according to the instructions of the manufacturer. The 30 µl reactions contained fixed amount of RNA (130 ng from spruce and 150 ng from Avicel cultivations), 400 U Superscript III reverse transcriptase (Invitrogen), 4 µl of 5× first strand buffer, 0.6 µM 3′-RACE primer, 0.6 µM SMART II oligonucleotide, 13 mM dithiothreitol (DTT) (Fermentas) and 1.3 mM dNTPs (Fermentas).

2.4. Real-time quantitative reverse transcription PCR (RT-qPCR)

The relative amount of cellulose-acting enzymes encoding gene transcripts was determined with RT-qPCR from the spruce wood and Avicel cultures of *D. squalens* after 7, 14, 21 and 28 d, and after

Please cite this article in press as: Rytioja, J., et al. Transcriptional analysis of selected cellulose-acting enzymes encoding genes of the white-rot fungus *Dichomitus squalens* on spruce wood and microcrystalline cellulose. Fungal Genet. Biol. (2014), http://dx.doi.org/10.1016/j.fgb.2013.12.008