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Differential regulation of laccase gene expression in *Coriolopsis rigida* LPSC No. 232

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ARTICLE INFO

Article history:
Received 30 July 2010
Received in revised form
10 September 2010
Accepted 16 September 2010
Available online 7 October 2010
Corresponding Editor:
Daniel C. Eastwood

Keywords:
Gene
Laccase
qRT-PCR
Transcriptional regulation
White-rot fungus

ABSTRACT

Two laccase isoenzyme genes (*lcc2* and *lcc3*) from the white-rot fungus *Coriolopsis rigida* were cloned, and together with the previously described *lcc1*, their transcript levels were analysed by Quantitative RT-PCR in order to study their expression patterns under a range of putative inducers (Cu^{2+}, Mn^{2+}, Fe^{3+}, 2,6-dimethoxy-1,4-benzoquinone, H_2O_2, caffeine, amphotericin B and syringic acid). The highest induction was observed for *lcc1* in presence of copper, and thus, a kinetic study was performed to analyze its effect on temporary *lcc1* gene expression. Our results showed that upregulation due to copper was linked to growth stage, being highest during the trophophase and decreasing during the idiophase. Amphotericin B increased levels of transcripts of *lcc1* and *lcc2*, syringic acid upregulated *lcc1* and *lcc3* and 2,6-dimethoxy-1,4-benzoquinone induced *lcc2* and *lcc3*. Possible reasons for why laccase genes from *C. rigida* are differentially regulated at the transcriptional level are discussed.

Introduction

Laccases (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) are phenol-oxidases belonging to the group of copper containing enzymes, known as blue oxidases (Messerschmidt & Huber 1990). They catalyze the oxidation of a great variety of phenolic compounds and aromatic amines using molecular oxygen as electron acceptor, and thus participating in the production of active oxygen species (Guillén *et al.* 2000) and in the degradation and transformation of lignin and soluble phenols as well as other xenobiotics (Paszczynski & Crawford 1995; Watanabe 2001; Asgher *et al.* 2008). Due to their action over a broad range of substrates, laccases have been widely studied for their potential use in several industrial applications, including pulp bleaching in paper industry, dye decolourisation, detoxification of environmental pollutants and revalorization of wastes and wastewaters (Mayer & Staples 2002; Saparrat *et al.* 2002; Jurado *et al.* 2009). Though laccases are widely distributed among plants, insects and some bacterial species, white-rot fungi have been considered as model organisms for their study and production (Martínez *et al.* 2005; Alcalde *et al.* 2007). In most of fungi, laccases have been found to be encoded by multigene

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1878-6146/$ – see front matter
doi:10.1016/j.funbio.2010.09.010

families (Valderrama et al. 2003). This gene redundancy, which usually involves differences in their physico-chemical and kinetic properties, regulatory mechanisms and localization (Crowe & Olsson 2001; Missall et al. 2005), suggest differential physiological roles such as ones related to nutrition, morphogenesis and inter-reaction. Although several studies have analysed the effects of culture parameters on laccase production, specially in order to enhance the yields for industrial applications, there is still some lack of knowledge about the regulation of laccase genes at the molecular level.

Coriolopsis rigida is a white-rot fungus that in the last two decades is being extensively studied for its ability to degrade lignin and several hazardous aromatic pollutants as well as to detoxify and revalorize organic wastes (Sampedro et al. 2004; Gómez et al. 2006; Saparrat et al. 2010). On several *in-vitro* culture conditions, this fungus secretes two laccases as the only ligninolytic enzyme component, which has been characterized in relation to several physico-chemical and catalytic properties (Saparrat et al. 2002). Recently it has been reported that these two laccases, encoded by *lcc1* gene, are directly involved in the transformation of aromatic compounds from a solid waste generated during the olive oil production (Díaz et al. 2010). The study of transcriptional regulation of this gene as well as other laccase genes in *C. rigida* is important to get a better insight into the oxidative system of this fungus and the physiological roles in which different laccases can be involved. Therefore, the objectives of this research were to identify laccase gene sequences in *C. rigida* and to investigate their gene expression by quantitative RT-PCR (qRT-PCR) under the effect of potentially laccase-inducing compounds. We also performed a kinetic study to analyze the effect of Cu^{2+} on the temporary laccase synthesis.

Materials and methods

Fungal material

Coriolopsis rigida strain LPSC No. 232 (Culture collection of the La Plata Spegazzini Institute = Spanish Type Culture Collection, CECT, no 20449), isolated from decaying wood collected in a subtropical Argentine rain forest (Ibáñez 1998), was maintained on a potato dextrose agar medium (Scharlau Chemie, Barcelona, Spain) at 4 °C.

Preparation of genomic DNA and PCR amplification

Genomic DNA was extracted from mycelial pellets collected from a glucose–peptone liquid medium (Saparrat et al. 2002) using Genomix DNA extraction kit (Talent, Italy), according to manufacturer's instructions.

Degenerate primers Pcu_1 (5′ CAYTGGCAYGGNTTYTTYCA 3′) and Pcu_4 (5′ TGRAARTCDATRTGRCARTG 3′), based on the conserved sequences of the copper-binding regions I (HWGGFFQ) and IV (HCHIDFH) and previously described (Hong et al. 2007), were used to amplify laccase sequences from *Coriolopsis rigida*.

PCR was performed in 1× PCR amplification buffer (Applied Biosystems) with 1 mM $MgCl_2$ (Applied Biosystems), 1 μM of each primer, 50 μM of each deoxynucleoside triphosphate (Promega), 0.2 μg of DNA template and 1.2 U of Taq DNA polymerase (Applied Biosystems) in a final volume of 50 μL, using a GeneAmp PCR System 2400 (PerkinElmer). Cycling parameters were 95 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 52 °C for 40 s and 72 °C for 1 min and the final extension at 72 °C for 10 min. Control reactions lacking template DNA were performed in parallel. Amplified fragments were visualized on 1 % agarose gels stained with ethidium bromide. PCR products were run in 1 % agarose gel and subsequently cut out, purified by a DNA gel extraction kit (Gene-clean, Q-BIOgene) and then inserted into pGEM-T easy cloning vector (Promega). After transformation of the recombinant vectors into the *Escherichia coli* DH5α strain, clones containing the inserted fragments were screened by DNA sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems).

Incubation of Coriolopsis rigida *mycelium with putative laccase inducers*

A mycelial suspension (1 %, v v^{-1}) was inoculated on a glucose–peptone liquid medium with agitation (150 rpm) at 28 °C (Saparrat et al. 2002). After 5 d of incubation, mycelial pellets were separated by filtration, washed with sterilized distilled water and 164 ± 12 mg (dry mass) was transferred to flasks containing 50 mL of 50 mM potassium phosphate buffer, pH 5.0, supplemented with the following potential laccase gene inducers: 300 μM Cu^{2+} (as $CuSO_4$ $5H_2O$), 150 μM Mn^{2+} (as $MnSO_4$ H_2O), 100 μM Fe^{3+} (as $FeCl_3$ $6H_2O$), 500 μM 2,6-dimethoxy-1,4-benzoquinone (DQ), 500 μM DQ + 100 μM Fe^{3+}, 500 μM H_2O_2, 5 mM caffeine, 1.5 μM amphotericin B and 1 mM syringic acid. Flasks were then incubated at 28 °C and 150 rpm for 15 h. Mycelium incubated in buffer under the same conditions but with no addition of inducers was used as control. Three replicates were used per treatment. After incubation, mycelia were separated from the buffer by filtration, and divided in two fractions: one used for RNA extraction and the other one to obtain a cell-free extract where laccase activity (considered as mycelium-associated activity) and proteins were measured as described below. The extract was prepared according to Günther et al. (1998) but with no addition of reduced glutathione in the extraction buffer. Extracellular laccase activity and proteins were also measured in the incubation liquids as described below.

Preparation of total RNA and reverse transcription

Mycelium pellets incubated for 15 h in control buffer or in the presence of several putative laccase inducers were washed with sterilized distilled water, and total RNA was isolated using Ultraspec RNA kit (Biotecx Laboratories, Inc.) and treated with DNAse I using the 'Deoxyribonuclease I, Amplification Grade' (Invitrogen, UK) to remove the DNA contamination from the samples. First strand complementary DNA (cDNA) was synthesized using the 'GeneAmp Gold RNA PCR Reagent Kit' (Applied Biosystems, USA). This cDNA was used as template in PCR reactions described above.

Quantitative RT-PCR

The sequences amplified by PCR using degenerate primers for the conserved sequences of the copper-binding regions and