Differential regulation of laccase gene expression in *Pleurotus sajor-caju*

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Four laccase isozyme genes, Psc lac1, 2, 3 and 4 have been cloned from the edible mushroom, *Pleurotus sajor-caju*. The genes display a high degree of homology with other basidiomycete laccases (55–99%) at the amino acid level. Of the laccase genes isolated, Psc lac1 and 4 displayed the highest degree of similarity (85% at the amino acid level), while Psc lac3 showed the highest degree of divergence, exhibiting only 52–57% amino acid similarity to the other *Pl. sajor-caju* laccase gene sequences. Laccase activity in *Pl. sajor-caju* is affected by nutrient nitrogen and carbon, and by the addition of copper and manganese to the growth medium. In addition, 2,5-xylidine, ferulic acid, veratric acid and 1-hydroxybenzotriazole induced laccase activity in the fungus. Induction of individual laccase isozyme genes by carbon, nitrogen, copper, manganese and the two aromatic compounds, 2,5-xylidine and ferulic acid, occurred at the level of gene transcription. While Psc lac3 transcript levels appeared to be constitutively expressed, transcript levels for the other laccase isozyme genes, lac1, 2 and 4, were differentially regulated under the conditions tested.

**Keywords:** white-rot fungus, transcriptional regulation, ligninolytic enzymes, polyphenol oxidases

**INTRODUCTION**

Laccase is a copper-containing polyphenol oxidase (EC 1.10.3.2) first discovered in the Japanese lacquer tree, *Rhus vernicifera* (Yoshida, 1883) over 100 years ago. It is structurally and evolutionarily related to the large blue copper protein group, which includes the plant ascorbate oxidases, and the mammalian plasma protein ceruloplasmin (Mayer, 1987; Reinhammer, 1984). It is a common enzyme and has been found to be widely distributed in plants (Mayer, 1987) and fungi (Hatakka, 1994). Laccase is dependent on four copper ions, which are distributed among three different highly conserved binding sites, for its function, with each copper ion appearing to play an important role in the catalytic mechanism (Reinhammer, 1984; Thurston, 1994). It catalyses the four-electron reduction of oxygen to water and this is typically accompanied by the oxidation of a phenolic substrate.

The biological role for laccase has yet to be fully elucidated and appears to vary depending on the type of organism (Thurston, 1994). In fungi, laccase has been well documented to act as a ligninolytic enzyme (Eggert *et al*., 1998); it is associated with pigment synthesis and sporulation in *Lentinus edodes* (Leatham & Stahman, 1981), with fruiting body formation in *Agaricus bisporus* (Wood, 1980) and *Schizophyllum commune* (De Vries *et al*., 1986), and with the pathogenicity of the chestnut blight fungus *Cryphonectria parasitica* (Choi *et al*., 1992). The enzyme also has applications in the food industry where laccase plays a role in tea and coffee fermentations and in vinification (Lante *et al*., 1992).

To more fully understand the function of laccases, several laccase genes, either as genomic or cDNA clones, have been isolated and characterized, including those from *Coriolus hirsutus* (Kojima *et al*., 1990), *Trametes versicolor* (Hunolstein *et al*., 1986; Jonsson *et al*., 1995, 1997), *Phlebia radiata* (Saloheimo *et al*., 1991), *Trametes villosa* (Yaver & Golightly, 1996; Yaver *et al*., 1996), the ligninolytic basidiomycete PM1 (Coll *et al*., 1993),

**Abbreviations:** EF, extracellular fluid; FA, ferulic acid; gDNA, genomic DNA; HBT, 1-hydroxybenzotriazole; HC, high carbon; HN, high nitrogen; LNC, low nitrogen and carbon; XYL, 2,5-xylidine.

The GenBank accession numbers for the sequences determined in this work are AF297525–AF297528.
Trametes I-62 (ECT 20197) (Mansur et al., 1998), Py. cinnabarinus (Eggert et al., 1997), Neurospora crassa (Germann et al., 1998), Aspergillus nidulans (Aramaya & Timberlake, 1990) and Rhizoctonia solani (Wahleithner et al., 1996). These genes typically display a high degree of identity with one another. In addition, the one cysteine and ten histidine residues, involved in binding the four copper atoms found in the majority of laccase molecules, are conserved, together with a small region around each of the four regions in which the copper ligands are clustered (Thurston, 1994). Several fungi code for more than one non-allelic variant, explaining in part the biochemical diversity of laccases. Differential regulation of laccase isozymes has been demonstrated in Trametes I-62 CECT 20197 (Mansur et al., 1998), Pleurotus eryngii (Muñoz et al., 1997), Lentinula edodes (Zhao & Kwan, 1999) and recently in Pleurotus ostreatus (Palmieri et al., 2000).

In this study we examined the effect of different physiological conditions on extracellular laccase production and on laccase isozyme regulation in Pleurotus sajor-caju. Previous studies have shown that there to be five laccase isoforms in Pl. sajor-caju (Fu et al., 1997). Using degenerate PCR primers based on conserved regions within the copper-binding sites of previously sequenced basidiomycete laccases, we cloned and sequenced four unique laccase isozyme genes from the fungus. Using competitive RT-PCR we demonstrate that differential expression occurs between individual isozyme genes, with some being constitutively expressed whilst others are induced under different physiological conditions. In addition, we demonstrate that induction of individual laccase isozyme genes by carbon, nitrogen, copper, manganese and the two aromatic compounds, 2,5-lactam (XYL) and 4-hydroxy-3-methoxycinnamic acid (ferulic acid; FA) occurs at the level of gene transcription.

METHODS

Organisms. The basidiomycete used in this study, Pl. sajor-caju P32-I, was obtained from J. Peberdy at the University of Nottingham, UK, and was maintained at 4 °C on glucose-malt extract (5 g glucose, 3.5 g malt extract and 15 g agar per litre).

Culture conditions. The basal medium (low nitrogen and carbon, LNC) contained, per litre, 10 g glucose (C_{6}H_{12}O_{6}, H_{2}O), 1 g ammonium tartrate (C_{4}H_{6}N_{2}O_{5}), 2.92 g 2,2’-dimethylsuccinate, 50 mg KH_{2}PO_{4}, 20 mg MgSO_{4}, 7H_{2}O. The medium was adjusted to pH 6.0 with 1 M NaOH, autoclaved and cooled before the addition of 2 mg thiamin and 10 ml trace elements solution, which contained, per litre, 1.5 g nitrotriacetate, 0.5 g MnSO_{4}, 5H_{2}O, 0.1 g NaCl, 0.1 g FeSO_{4}, 7H_{2}O, 0.1 g CuSO_{4}, 5H_{2}O, 0.1 g CoCl_{2}, 0.1 g ZnSO_{4}, 7H_{2}O, 0.01 g Na_{2}MoO_{4}, 2H_{2}O and 0.01 g AlK(SO_{4})_{2}. Three agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a glucose-malt extract plate (6–8 d) were used as the inoculum. The fungus was grown in 15 ml stationary cultures in 200 ml medical flat bottles (BDH) at 26 °C in darkness. Bottles were loosely capped to allow passive aeration. To determine the point of maximal laccase production, a time course experiment in which laccase activity was measured over a period of 22 d was conducted. To determine the effects of nutrient nitrogen, carbon, manganese and copper on laccase activity and gene expression, the LNC medium was supplemented with either ammonium tartrate [50 mM, high nitrogen (HN)], glucose [60 mM, high carbon (HC)], manganese as MnSO_{4} and copper as CuSO_{4} to a final concentration of 300 µM. Laccase activity was measured over 6 d and the mycelia harvested for RNA extraction and RT-PCR analysis. To determine the effect of several aromatic compounds on laccase activity and transcription, these compounds were filter-sterilized and added on the sixth day of cultivation. The aromatic compounds added were XYL, 1-hydroxybenzotriazole (HB1), 3,4-dimethoxybenzoic acid (veratric acid) and FA. These were added to a final concentration of 300 µM, the laccase activity was measured after 48 h and mycelia samples taken. Laccase activities were determined with 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the substrate (Wolfenden & Willson, 1982). Datum points in all cases are means for triplicate cultures, with standard deviations indicated by error bars.

Design of PCR primers. Primers (Plac) corresponding to the highly conserved copper-binding regions of previously sequenced basidiomycete laccase genes were used to isolate laccase-gene-specific sequences from Pl. sajor-caju. These PCR products were cloned into the PCR 2.1 Topo vector (Invitrogen). Sequence data from these clones revealed three unique laccase isozymes; subsequently, a fourth isozyme gene was cloned from a Pl. sajor-caju cDNA library (Stratagene), with primers (Plc L1) designed from the Pl. ostreatus PoX1 sequence (Giardina et al., 1995). RNA for the construction of this library was obtained from mycelia grown under basal conditions (LNC) as previously described but in the presence of 300 µM CuSO_{4} and 200 µM XYL, with the RNA being harvested 6 d after inoculation. Primers for the housekeeping gene β-tubulin were designed based on GenBank sequence data. The sequence of the primers used and the sizes of the corresponding cDNA and genomic DNA (gDNA) products in addition to the annealing temperatures used are listed in Table 1.

DNA sequencing and analysis. Sequencing of putative laccase genes was determined by the dyeoxygen chain-termination method (Sanger et al., 1977) using the Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase, FS (Applied Biosystems), on a GeneAmp PCR system 2400 (Perkin Elmer), and run on an automated DNA sequencer (model 373 stretch, Applied Biosystems). The sequence data were assembled and processed using the DNASTAR (DNASTAR Inc.) software package. The BLAST algorithm (Altschul et al., 1990) was used to search DNA and protein databases for similarity. The CLUSTAL program was used for alignment of amino acid sequences.

RNA preparation. Total RNA was prepared by a modification of the method of Gromroff et al. (1989). Mycelia from triplicate cultures were separated from culture fluid by filtering through Miracloth (Calbiochem), washed twice with distilled water, quick frozen in liquid nitrogen, and ground to a powder with a mortar and pestle. Lysis buffer [0.6 M NaCl, 10 mM EDTA, 100 mM Tris/HCl (pH 8), 4% SDS and 50% phenol] was added and the mixture was shaken vigorously for 20 min. It was then centrifuged for 10 min at 14000 g. After a further extraction step with phenol/chloroform/isoamyl alcohol (25:24:1), 0.75 vol. 8 M LiCl was added and the mixture vortexed and incubated overnight at 4 °C. RNA was pelleted by centrifugation for 15 min at 14000 g and resuspended in...
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**Table 1. Gene-specific primer sequences and annealing temperatures used for competitive RT-PCR**

<table>
<thead>
<tr>
<th>Primer/ gene*</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
<th>Annealing temperature (°C)</th>
<th>Size (bp)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plac</td>
<td>CATTGGCATGGCTTCTTCCA</td>
<td>GCGGAATCCAGTAATGGCGAC</td>
<td>52.0</td>
<td>594</td>
</tr>
<tr>
<td>Psc lac1</td>
<td>CGATCCCAACCTGTCCTTG</td>
<td>GGTCTGCCCTGATGACC</td>
<td>61.5</td>
<td>384</td>
</tr>
<tr>
<td>Psc lac2</td>
<td>GCTTCCAGTCAAGCTT</td>
<td>ATGCTAGTGGAGACGT</td>
<td>61.5</td>
<td>476</td>
</tr>
<tr>
<td>Psc lac3</td>
<td>GAAAGGAAGCAGCTGG</td>
<td>CGAGGGGAACTGATCAGCC</td>
<td>61.5</td>
<td>52</td>
</tr>
<tr>
<td>Psc lac4</td>
<td>CGACCCGCAGATCTTCTT</td>
<td>GAGGCGATATTGTGGCATC</td>
<td>61.5</td>
<td>565</td>
</tr>
<tr>
<td>Psc β-tubulin</td>
<td>TTGATGCGTCTCCTGATGTTTGTG</td>
<td>AGCGGGGATATTGTGGCATC</td>
<td>61.5</td>
<td>565</td>
</tr>
<tr>
<td>Psc L1</td>
<td>GATATTTTCAGGCGACCG</td>
<td>GCGGGGATATTGTGGCATC</td>
<td>52.0</td>
<td>1436</td>
</tr>
</tbody>
</table>

*GenBank accession numbers are as follows: Psc lac1, AF297525; Psc lac2, AF297526; Psc lac3, AF297527; Psc lac4, AF297528; Psc β-tubulin, AF008134.

† Size predicted for PCR amplification products with cDNA and gDNA templates.

Water. It was precipitated with CH₃COONa (pH 5.5) and 99% ethanol, washed with 70% ethanol, and resuspended in water. Residual contaminating DNA was removed by digestion with DNase I (Boehringer) according to the manufacturer’s protocol. Total RNA was quantified spectrophotometrically at 260 and 280 nm.

**RT-PCR.** Total RNA was used as the template to generate first-strand cDNA in reaction mixtures containing 1-5 µg RNA, 600 ng random hexamer primers (Boehringer), 0.5 mM (each) deoxynucleoside triphosphates, 20 U RNASin ribonuclease inhibitor (Promega), 10 mM DTT, 1 µM Expand reverse transcriptase buffer (Boehringer) and 50 U Expand reverse transcriptase (Boehringer). Reaction volumes were adjusted to 20 µl with RNase-free water. Reaction mixtures were incubated at 30 °C for 10 min followed by 42 °C for 45 min. Heating to 93 °C for 5 min terminated reactions. For PCR amplification, a 2 µl volume from each RT reaction mixture was mixed with 10 pmol each of laccase-isozyme-specific primer (Table 1), 2 µl 10×NH₄Cl-Taq buffer (Bioline), 1.5 mM MgCl₂, 100 µM (each) deoxynucleoside triphosphates and 1.25 U Taq polymerase. Reaction volumes were adjusted to 20 µl with HPLC-grade water. Amplification was performed in a PTC-100 programmable thermal controller (MJ Research) with 1 cycle of 96 °C for 5 min followed by 33 cycles of denaturation (45 s at 94 °C), annealing (45 s at 61.5 °C) and extension (45 s at 72 °C), with a final extension of 72 °C for 10 min. The number of cycles used in the PCR was varied to avoid reaching a point at which bands representing different conditions would have equal intensities due to reaching a plateau in amplification. Following amplification, 20 µl of each PCR product was loaded on a 2% agarose gel and electrophoresed in TAE buffer (40 mM Tris/acetate, 1 mM EDTA) for 2 h at 90 V. The gel was stained with ethidium bromide, visualized under UV light and photographed. Each cDNA PCR product was subsequently sequenced to confirm its identity.

**Quantiﬁcation of competitive RT-PCR products.** Photographs of the ethidium bromide-stained gels were scanned and densitometry was performed (ImageQuaNT software; Molecular Dynamics). The transcript concentrations were calculated by determining the concentration at which the competitor gDNA and cDNA targets were equal. To correct for differences in molecular mass, the intensity of the competitor band was multiplied by a factor of cDNA (bp)/gDNA (bp). The log₁₀ of the cDNA/gDNA ratio was plotted as a function of the log₁₀ of the initial amount of competitor (Piatka et al., 1993; Siebert & Larrick, 1992). The initial amount of cDNA was calculated by extrapolating from the intersection of the curves, where the amounts of target and competitor are equal (point of equivalence, ratio = 1, log₁₀ 1 = 0), to the x axis. The relative increase for each gene upon varying culture conditions should take into account the fact that an indirect method is used in the quantification, and therefore the values given should not be taken as absolute.

**RESULTS**

**Laccase activity in Pl. sajor-caju culture**

A time course for laccase activity in the extracellular fluid (EF) of *Pl. sajor-caju* grown in LNC culture is shown in Fig. 1(a). Laccase activity was first detected on day 2 and reached a peak after 6 d; thereafter it decreased, reaching an almost negligible level by day 22. The pH optimum for laccase in EF was 5.5 with a sharp decline below pH 5.0 (data not shown).

**Effect of different physiological growth conditions on laccase production**

Fig. 1(b) shows the extracellular laccase activity of *Pl. sajor-caju* grown in stationary culture under LNC, or LNC with HC, HN, high manganese (Mn) or high copper (Cu), and assayed after 6 d. Enzyme levels in LNC cultures reached a peak [2.6 U (mg protein)⁻¹] after 6 d, whilst corresponding cultures of HC and HN were slightly higher [2.9 and 3.6 U (mg protein)⁻¹, respectively].

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On: Mon, 14 Jan 2019 16:40:13
In LNC cultures in the presence of 300 µM MnSO₄, the laccase activity increased from 2.64 to 4.12 U (mg protein)⁻¹ when compared to LNC cultures containing no added manganese. Similarly, the addition of 300 µM CuSO₄ to the LNC cultures resulted in a 3.7-fold increase in specific activity after 6 d growth. Above 300 µM, both copper and manganese significantly inhibited fungal growth. The transition metal zinc added as ZnSO₄ had no detectable effect on laccase activity at 300 µM (data not shown).

Fig. 1(c) illustrates the effect on laccase activity in *Pl. sajor-caju* when the fungus was grown in the presence of various concentrations of XYL, HBT, FA and veratric acid. XYL (300 µM), 100 µM HBT and 500 µM veratric acid resulted in 21.1-, 16.4- and 5.9-fold increases respectively, compared to the basal LNC culture with no additions. FA showed a 10.5-fold increase in activity at 100 µM; the change in specific activity, however, was much lower (1.7-fold increase) due to the large increase in the protein concentration (5.5 to 29.8 µg µl⁻¹). The presence of 300 µM or higher concentrations of all four aromatic compounds tested resulted in inhibition of fungal growth, indicating that higher concentrations of these compounds were toxic to the fungus.

### Isolation and analysis of laccase isozyme gene sequences

To isolate laccase isozyme genes from *Pl. sajor-caju*, we designed PCR primers based on conserved copper-binding domains of previously cloned basidiomycete laccase genes. The PCR was carried out over several annealing temperatures; the PCR product formed at the lowest temperature (52 °C) in which a single band (1.0 kb) was observed was subsequently cloned. Eight clones were sequenced and three putative laccase isozyme genes were identified and assigned the names Psc lac1, 2 and 3. An additional laccase isozyme gene, Psc lac4, was also cloned from a *Pl. sajor-caju* cDNA library using primers (Psc L1, Table 1) based on sequence from the Pox 1 gene (Girardina et al., 1995). The predicted amino acid sequence of the isozyme from the four cloned genes from *Pl. sajor-caju* had a high degree of similarity to the corresponding sequences of other basidiomycete laccase genes (55–99% identity at the amino acid level), from which the degenerate primers were designed: *Ph. radiata*, *Pl. ostreatus*, *T. versicolor*, *T. villosa* and *Trametes* I-62 (CECT 20197) (Fig. 2). Similarity to the corresponding sequences in the laccase genes of the ascomycete fungi *A. nidulans* and *N. crassa* was lower (20–50% identity). The isozymes showed 20–30% identity at the nucleotide level to the *Cucumis sativus* ascorbate oxidase, another member of the blue copper protein group. The Psc lac1 isozyme had a 93% nucleotide identity (99.5% at the amino acid level) with the Pox 1 gene in *Pl. ostreatus*. Psc lac4 was found to be 97% identical at the nucleotide level (99.1% at the amino acid level) to the Pox 2 gene of *Pl. ostreatus*. Psc lac3 showed the highest degree of divergence of the four laccase isozymes cloned, exhibiting only 52–57% identity with the other three isozymes. All the isozymes showed the highest conservation with each other in the copper-binding domains.

The positions of introns in the Psc lac1, 2, 3 nucleotide sequences were inferred initially from the consensus sequences for the 5′ and 3′ splicing sites of other eukaryotes, (Balance et al., 1986). This enabled primers to be designed around putative exon regions. PCRs were carried out on cDNA and gDNA and the products formed were sequenced. These data allowed us to design further primers inside the exon regions for subsequent RT-PCR analysis of the isozymes (Table 1).
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Fig. 2. Comparison of the *Pl. sajor-caju* laccase isozyme amino acid sequences with various basidiomycete laccase amino acid sequences. White letters on a dark background indicate common amino acids. The positions of two pairs of histidine amino acids proposed to be involved in Cu$^{2+}$ binding (Thurston, 1994) are indicated with black circles. Cysteine residues putatively involved in forming disulfide bridges (Jönsson et al., 1995) are indicated by black squares. The amino acid sequences were either experimentally determined or deduced from nucleotide sequences of *Psc lac1* (*P. sajor-caju*), *Psc lac2* (*P. sajor-caju*), *Psc lac3* (*P. sajor-caju*), *Psc lac4* (*P. sajor-caju*), *Pl. ostreatus* *Pox1* (*Pox1*) (Giardina et al., 1995), *Pl. ostreatus* *Pox2* (*Pox2*) (Giardina et al., 1999), *Ag. bisporus* laccase (*A. bisporus lcc1*) (Smith et al., 1998), basidiomycete I-62 laccase (Cect lac1) (Mansur et al., 1997), *Ph. radiata* laccase (Pr lac) (Saloheimo et al., 1991), *T. versicolor* laccase (T. var lcc1) (Jönsson et al., 1995) and *T. villosa lcc2* (T. var lcc2) (Yaver et al., 1996).

RT-PCR analysis of laccase isozyme mRNA transcripts

Initial RT-PCR analysis demonstrated that the β-tubulin and the Psc lac3 genes were expressed constitutively under all the conditions tested (Fig. 3a and d, respectively). Variations in transcript levels were observed with *Psc lac1*, *Psc lac2* and *Psc lac4*, with all showing a reduction after day 6 (Fig. 3b, c and e, respectively). Nitrogen, carbon, copper, manganese, FA and XYL all appeared to induce transcription to varying degrees relative to similar time points in LNC cultures (days 6, 8 and 10) (Fig. 3). Competitive RT-PCR was then performed to determine the changes in transcript levels, for each of the four isozymes and the housekeeping gene β-tubulin with transcript concentrations being expressed as molecules per ng total RNA (Table 2).

The housekeeping gene β-tubulin was expressed approximately 100-fold higher than any of the laccase isozyme genes under LNC growth conditions and was constitutively expressed [mean 2.24 ± 0.03 × 10^8 molecules (ng total RNA)^−1] (Table 2). Similarly, *Psc lac3* transcript
levels were largely unaffected under the conditions tested, giving a mean of $2.13 \pm 0.1 \times 10^3$ molecules (ng total RNA)$^{-1}$ (Table 2). Transcript levels for the other laccase isozymes were differentially regulated under the conditions tested. Psc lac1 transcript levels increased 204-, 427- and 15-fold in the presence of HC, Cu and Mn, respectively when compared to similar time points in LNC cultures (Table 2). HN, HC, Cu, Mn, FA and XYL increased Psc lac2 transcript levels 159-, 118-, 430-, 21-, 515- and 602-fold, respectively (Table 2). Similarly, Psc lac4 transcript levels increased 300-, 287-, 552-, 30-, 638- and 745-fold in the presence of HN, HC, Cu, Mn, FA and XYL, respectively (Table 2).

**DISCUSSION**

In this study, we report on the cloning of four unique laccase isozyme genes from *Pl. sajor-caju* which display a high degree of similarity with other published basidiomycete laccases (Fig. 2). The deduced amino acid sequences of Psc lac1 and 4 showed the highest degree of homology (88.3% identity) of the isozymes cloned, whilst Psc lac3 exhibited the lowest identity to the other isozymes (53–57%). The genes have some identity with the ascomycete laccases (24–62%) but fairly low identity (12–16%) with the laccase (yA) from *A. nidulans* (Aramayo & Timberlake, 1990). In fungi, besides lignin degradation (Eggert et al., 1998; Hatakka, 1994; Thurston, 1994), laccases have been implicated in different biological processes such as in *A. nidulans* where the product of the yA laccase gene has been shown to be uniquely involved in the formation of a green pigment in the conidium (Thurston, 1994). In *Ag. bisporus*, laccase appears to be involved in ligninolytic growth (Wood, 1980) and studies have also suggested roles for laccase in fruiting body formation (Leatham & Stahman, 1981), sporulation and in plant pathogenesis (Choi et al., 1992). Therefore, it is possible that different isozymes have evolved in fungi to allow them to perform different functional roles. However, it remains to be

**Table 2. Quantification of transcript levels for Psc lac1, 2, 3 and 4 and β-tubulin under various culture conditions**

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Psc lac1</th>
<th>Psc lac2</th>
<th>Psc lac3</th>
<th>Psc lac4</th>
<th>β-Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6 (LNC)</td>
<td>$6.41 \times 10^4$</td>
<td>$7.61 \times 10^4$</td>
<td>$2.08 \times 10^4$</td>
<td>$7.06 \times 10^4$</td>
<td>$2.21 \times 10^4$</td>
</tr>
<tr>
<td>Day 8 (LNC)</td>
<td>$4.56 \times 10^4$</td>
<td>$3.65 \times 10^4$</td>
<td>$2.01 \times 10^4$</td>
<td>$4.80 \times 10^4$</td>
<td>$2.26 \times 10^4$</td>
</tr>
<tr>
<td>Day 10 (LNC)</td>
<td>$1.64 \times 10^4$</td>
<td>$1.73 \times 10^4$</td>
<td>$2.21 \times 10^4$</td>
<td>$1.69 \times 10^4$</td>
<td>$2.25 \times 10^4$</td>
</tr>
<tr>
<td>HN</td>
<td>$5.78 \times 10^4$</td>
<td>$1.22 \times 10^4$</td>
<td>$2.35 \times 10^4$</td>
<td>$2.12 \times 10^4$</td>
<td>$2.34 \times 10^4$</td>
</tr>
<tr>
<td>HC</td>
<td>$1.31 \times 10^4$</td>
<td>$9.00 \times 10^3$</td>
<td>$2.38 \times 10^4$</td>
<td>$2.03 \times 10^4$</td>
<td>$2.28 \times 10^4$</td>
</tr>
<tr>
<td>Cu</td>
<td>$1.95 \times 10^4$</td>
<td>$1.57 \times 10^4$</td>
<td>$2.17 \times 10^4$</td>
<td>$2.65 \times 10^4$</td>
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</tr>
<tr>
<td>Mn</td>
<td>$7.02 \times 10^4$</td>
<td>$7.92 \times 10^4$</td>
<td>$2.12 \times 10^4$</td>
<td>$1.46 \times 10^4$</td>
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<tr>
<td>FA</td>
<td>$3.58 \times 10^3$</td>
<td>$1.88 \times 10^4$</td>
<td>$1.97 \times 10^4$</td>
<td>$3.16 \times 10^4$</td>
<td>$2.22 \times 10^4$</td>
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<tr>
<td>XYL</td>
<td>$3.92 \times 10^3$</td>
<td>$2.20 \times 10^4$</td>
<td>$1.89 \times 10^4$</td>
<td>$3.58 \times 10^4$</td>
<td>$2.19 \times 10^4$</td>
</tr>
</tbody>
</table>

* HN, HC, Cu, Mn, FA and XYL represent high-nitrogen, -carbon, -copper, -manganese, -FA and -XYL growth conditions, respectively. Day 6 (LNC) is the control for HN, HC, Cu and Mn, whilst day 8 (LNC) is the control for XYL and FA. Day 10 (LNC) represents culture mRNA levels 10 d after inoculation.

† The standard error of quantifications was less than 5%.
determined whether multiple laccases that have different physiological functions exist within the same organism.

Nitrogen appears to regulate laccase expression in *Pl. sajor-caju*. HN increases the laccase activity in the EF (Fig. 1b) and competitive RT-PCR analysis of the isozymes under HN conditions shows that lac2 and 4 transcripts are approximately 16- and 30-fold higher compared to LNC transcripts (Table 2). A similar effect has been reported in the ligninolytic basidiomycete *I. 62* (CECT 20179) (Mansur et al., 1998) where lcc1 and lcc2 transcript levels increased 100-fold under HN culture conditions, whilst Eggert et al. (1998) have shown that laccase activities in the culture fluids of *Py. cinnabarinus* are dependent on the carbon : nitrogen ratio. It is possible that this nitrogen regulation of lac2 and 4 in *Pl. sajor-caju* may be mediated by a NIT2-like protein similar to that involved in nitrogen metabolite regulation in *N. crassa* (Feng & Marzluf, 1998), given that three NIT2-binding sites conforming to the consensus sequence, TATCT, (Jarai et al., 1992), are present in the sequence upstream from the TATA box in the lac4 gene. However, functional analysis studies will need to be performed on the promoter sequences of the respective isozymes before this can be proven.

Addition of high copper (300 µM CuSO₄) to *Pl. sajor-caju* cultures induces laccase activity in the EF and lac1, 2 and 4 transcript levels increased approximately 43-, 43- and 55-fold higher, respectively. Copper has previously been reported to increase the laccase activity in *N. crassa* (Huber & Lerch, 1987), and to increase laccase gene transcription in *T. versicolor* (Collins & Dobson, 1997) and *Pl. ostreatus* (Palmieri et al., 2000). How this effect is mediated is as yet unknown. A response element has been identified in the promoter region of the lac1 gene in the basidiomycete PM1 (CECT 2971) (Coll et al., 1993) which displays some similarity with the binding site for the ACE1 transcription factor in the *Saccaromyces cerevisiae SOD1* gene (Gralla et al., 1991). This gene encodes a Cu-Zn superoxide dismutase which is regulated by copper and zinc. A similar putative response element, containing 15 of the 21 nucleotides of the ACE1 consensus binding site, is present approximately 400 bp upstream from the TATA box of the lac4 gene. Another possibility is that free copper ions present in the growth media of *Pl. sajor-caju* could result in induction of laccase in a similar fashion to how yeast responds to copper ion toxicity through the production of the copper-chelator Cu-metallothionein (Cervantes & Gutiérrez-Corona, 1994), given that laccases contain binding sites for copper ions, which are essential for enzyme activity (Huber & Lerch, 1987).

Manganese also appears to increase both laccase activity and expression in *Pl. sajor-caju*. The addition of 300 µM MnSO₄ resulted in an approximate 1-5-fold increase in laccase activity (Fig. 1b), whilst lac1, 2 and 4 transcript levels increased 1-5-, 2-1- and 3-fold, respectively (Fig. 3; Table 2). Manganese is found in lignin, the natural substrate for white-rot fungi and its ability to induce manganese peroxidase (MnP) transcription is well established (Gold & Alic, 1993). Archibald & Roy (1992) have shown that the laccase from *T. versicolor* can produce Mn(III) chelates from Mn(II) in the presence of a phenolic ‘accessory’ and others have suggested a dual role for both laccase and MnP in lignin degradation; however, this work provides direct evidence suggesting a link between both enzymes at the level of induction. Similarly, recent work on the white-rot fungi *Clitocybula dusenii* and *Nematoloma frowardii* has also reported increased laccase mRNA levels in cultures supplemented with manganese (Scheel et al., 2000). Several putative metal response elements (MREs) have been identified in the promoter regions of MnP and laccase genes (Giardina et al., 1999; Gold & Alic, 1993). These putative MREs conform exactly to the consensus sequence found in the promoters of metallothionein genes in higher eukaryotes. A range of heavy metals induces the expression of these genes, with regulation operating via a metal-regulatory protein which functions both as a metal receptor and as a trans-acting transcription factor.

Aromatic compounds which are structurally related to lignin, such as XYL, FA or veratric acid, are routinely added to fungal cultures to increase laccase production (Collins & Dobson, 1997; Muñoz et al., 1997; Yaver et al., 1996). XYL is known to increase laccase transcription in both *T. villosa* (Yaver et al., 1996) and *T. versicolor* (Collins & Dobson, 1997). A similar inductive effect was observed in *Pl. sajor-caju*, where XYL, FA, veratric acid and HBT induced laccase activity in the EF (Fig. 1c), although at concentrations in excess of 2 mM no fungal growth occurred. It has been proposed (Thurston, 1994) that one of the possible functions for fungal laccases is in the polymerization of toxic aromatic compounds formed during the degradation of lignin. Therefore, laccases may function as a defence mechanism against oxidative stress. Laccase reactions, by consuming oxygen, are expected to disfavour redox cycling of quinones with oxygen and, whereas the autoxidation of hydroquinones and semiquinones leads to the generation of oxygen radicals, the corresponding laccase-catalysed oxidations yield water. Fernández-Larrea et al. (1996) reported that the oxidative stress in *Podospora anserina* such as that caused by the presence of aromatic compounds was typically accompanied by the induction of laccase mRNA. A dark precipitate has been observed in XYL-induced cultures of *T. versicolor* and it has been suggested it may represent a laccase-polymerized form of the aromatic compound (Collins & Dobson, 1997). We noted a similar precipitate in XYL- and to a certain extent in FA-induced cultures of *Pl. sajor-caju*. Two similar sites exactly matching the XRE consensus sequence TNGCGTG (Rushmore et al., 1991) are also present in the region upstream from the lac4 promoter in *Pl. sajor-caju*. The presence of these putative XRE elements suggests that transcription of laccase genes may be activated by aromatic compounds, such as those studied here, while the absence of putative XRE elements in the promoter regions of laccase genes in other fungal species may be due to the fact that these genes are not induced by aromatic compounds, or that
other, as yet unidentified, aromatic response element(s) could be present.

The Psc lac3 isozyme gene in Pl. sajor-caju is apparently constitutive, but whether it is mechanistically constitutive, in that it lacks promoter sequences for modulation of transcription, or is being induced by an as yet unidentified product of metabolism of the fungus, remains to be determined. For many of the potential putative response elements discussed above, such as XRE and MRE elements or NIT2-like protein-binding sites, however, it will remain uncertain as to whether any of them are in fact functional transcription factor recognition sites until either a suitable promoter reporter assay system or a reliable transformation system is developed for Pleurotus species.

In conclusion, the results presented here indicate that the Pl. sajor-caju laccase isozyme genes are differentially regulated at the transcriptional level in response to copper, manganese, nutrient nitrogen and other culture conditions. Further work is required to investigate the precise mechanism(s) of transcriptional activation of the laccase genes in this and other fungi in order to more fully understand the biological function of these individual isozymes.

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