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Transcriptional profiles of laccase genes in the brown rot fungus *Postia placenta* MAD-R-698

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One of the laccase isoforms in the brown rot fungus *Postia placenta* is thought to contribute to the production of hydroxyl radicals, which play an important role in lignocellulose degradation. However, the presence of at least two laccase isoforms in this fungus makes it difficult to understand the details of this mechanism. In this study, we systematically investigated the transcriptional patterns of two laccase genes, *Pplcc1* and *Pplcc2*, by quantitative PCR (qPCR) to better understand the mechanism. The qPCR results showed that neither of the two genes was expressed constitutively throughout growth in liquid culture or during the degradation of a woody substrate. Transcription of *Pplcc1* was upregulated under nitrogen depletion and in response to a high concentration of copper in liquid culture, and during the initial colonization of intact aspen wafer. However, it was subject to catabolite repression by a high concentration of glucose. Transcription of *Pplcc2* was upregulated by stresses caused by ferulic acid, 2, 6-dimethylbenzoic acid, and ethanol, and under osmotic stress in liquid culture. However, the transcription of *Pplcc2* was downregulated upon contact with the woody substrate in solid culture. These results indicate that *Pplcc1* and *Pplcc2* are differentially regulated in liquid and solid cultures. *Pplcc1* seems to play the major role in producing hydroxyl radicals and *Pplcc2* in the stress response during the degradation of a woody substrate.

Keywords: brown rot fungi, laccase, *Postia placenta*, qPCR, transcriptional regulation

Introduction

Laccases belong to a superfamily of multi-copper-containing oxidases whose members oxidize many kinds of phenolic compounds and aromatic amines through the simultaneous reduction of molecular oxygen to water (Giardina *et al*., 2010). Most laccases have been found in plants and fungi. They are thought to be involved in several important physiological processes such as lignin formation in plants, and in morphogenesis, stress defense responses, and pigmentation in fungi (Thurston, 1994; Lundell *et al*., 2010). White rot fungi are thought to mineralize lignin during the degradation of wood using lignin peroxidase, manganese peroxidase, and versatile peroxidase in combination with laccase. The laccases in white rot fungi have received special attention because they are thought to be involved in the oxidation of phenolic components alone, or in the oxidation of non-phenolic components with the help of small mediators derived from lignin (Kirk and Cullen, 1998; Morozova *et al*., 2007). The oxidation reaction has been suggested to aid in the depolymerization of lignin. The ability of laccases to oxidize recalcitrant lignin components using molecular oxygen as the electron receptor has huge potential applications in textile, biomass conversion, food, bioremediation, wastewater treatment, wood processing, pharmaceutical, and chemical industries (Couto and Herrera, 2006). Consequently, the identification, purification, production, biochemical characterization, and regulation mechanism of laccases in white rot fungi have been extensively investigated (Majeau *et al*., 2010; Villalba *et al*., 2010).

In brown rot fungi, another group of wood-rotting basidiomycetes, the depolymerization process is thought to be initiated by highly destructive hydroxyl radicals produced by extracellular Fenton chemistry (Jensen *et al*., 2001; Karripally *et al*., 2013). The reduction of iron is a prerequisite for this reaction; this reduction step is achieved by phenolic compounds such as 2, 5-dimethoxy-1, 4-benzoquinone or 4, 5-dimethoxyl-1, 2-benzoquinone (Enoki *et al*., 1997; Kerem *et al*., 1999). However, the high concentration of oxalate produced by most brown rot fungi during the initial colonization process inhibits the reduction reaction by chelating Fe^{3+} and decreasing the reducing potential of the trioxalate complex. Our previous study showed that laccase aids in this reduction process (Wei *et al*., 2010).

Many studies have focused on laccases from white rot fungi, but few have analyzed laccases from brown rot fungi (D'Souza *et al*., 1996; Wei *et al*., 2010; Kües and Rühl, 2011; Arantes *et al*., 2012). This is because laccase activity has rarely been detected in brown rot fungi using traditional substrates and several detection methods (Gigi *et al*., 1981). Therefore, brown rot fungi were thought to lack laccases, or to be poor laccase producers (Machuca and Ferraz, 2001; Arantes *et al*., 2012). However, data from recent genome sequencing projects have revealed that there are *sensu stricto* laccase sequences in most of the brown rot fungi investigated so far (Martinez *et al*., 2009; Floudas *et al*., 2012; Riley *et al*., 2014).

There are two explanations for the discrepancy between the presence of laccase sequences in the genome and the rarely

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detected laccase activity in brown rot fungi. First, laccases from brown and white rot fungi might have different catalytic abilities. However, *Pplcc1* (Protein ID 111314) from the model brown rot fungus *Postia placenta* was shown to encode a functional laccase that can oxidize traditional substrates such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), and syringaldazine (SGZ) (Wei *et al*., 2010). Recently, research in our laboratory confirmed that *Pplcc2* (Protein ID 62097) also encodes a functional laccase that can oxidize ABTS and DMP but not SGZ (unpublished data). These results imply that the laccase sequences in brown rot fungi encode multiple laccases with different catalytic abilities just like those from white rot fungi. The other explanation for the discrepancy might be that the transcriptional regulation mechanisms of laccases differ between white and brown rot fungi. At least one laccase is constitutively expressed in most white rot fungi (Piscitelli *et al*., 2011). However, few studies have focused on the regulation of laccase expression in the brown rot fungus *P. placenta*. Expression of *Pplcc1* in basal medium with cellulose was reported to be about 2.29-fold higher than that in medium with glucose as the carbon source, and the transcription of *Pplcc2* decreased slightly (Martinez *et al*., 2009). The expression of these two genes during the degradation of furfurylated Scots pine and untreated samples was investigated by qPCR, but the time course of expression was not reported (Alfredsen and Fossdal, 2009). Therefore, these studies did not systematically investigate the regulatory mechanism of laccase in *P. placenta*.

The second reason why brown rot fungi laccases have been a less attractive research topic is because laccases are not thought to be important for lignin degradation, even in white rot fungi. Two lines of evidence support this idea: first, the redox potential of laccases is too low to oxidize the non-phenolic units of lignin; and second, *Phanerochaete chrysosporium*, the model organism used in lignin degradation studies, was not thought to express laccase (Kirk and Farrell, 1987; De Jong *et al*., 1994; Hatakka, 1994). However, a recent review has provided several pieces of evidence supporting the deep involvement of laccase in the degradation of lignin by white rot fungi (Munk *et al*., 2015). Therefore, further research is required to clarify the roles of laccase in the degradation of lignin components (Giardina *et al*., 2010).

P. placenta is a well-studied model brown rot fungus with two functional laccase genes, *Pplcc1* and *Pplcc2*, in its genome (Martinez *et al*., 2009). *Pplcc1* is thought to play a role in the decay mechanism by helping to generate an extracellular Fenton system through oxidizing hydroquinone to semiquinone radicals (Wei *et al*., 2010). Semiquinone radicals derived from the oxidation reaction reduce ferric iron to ferrous ions and activate oxygen to produce hydrogen peroxide (Wei *et al*., 2010). The reaction between ferrous ions and hydrogen peroxide leads to the production of hydroxyl radicals, which are essential for the initial colonization of wood by both white and brown rot fungi (Goodell *et al*., 1997; Guillen *et al*., 2000). The proposed function of laccase in this fungus was supported by the purification and characterization of a recombinant PPLCC1 and simulation of the laccase/hydroquinone system *in vitro* (Wei *et al*., 2010). However, it is unknown which of the two laccase genes in the genome is the main contributor to this function. Knocking out the two laccase variants in *P. placenta* individually or together would confirm their role *in vivo* (Kües and Rühl, 2011). However, reliable methods for transforming *P. placenta* have not yet been developed. Until reliable transformation techniques are available for *P. placenta*, studies of transcriptional regulation can be used to unravel the different physiological roles of laccases in *P. placenta* and other wood rot fungi (Pezzella *et al*., 2013).

The aim of this study was to gain insight into the physiological functions of laccases in brown rot fungi. Many factors affect the transcriptional profiles of laccase genes in white rot fungi (Piscitelli *et al*., 2011; Janusz *et al*., 2013). Combinations of these factors might affect laccase gene transcription synergistically or antagonistically, which can complicate transcriptional analyses (Collins and Dobson, 1997; Galhaup *et al*., 2002; Piscitelli *et al*., 2011). The relationship between laccase activity and the transcriptional level of laccase genes is also complicated by several factors such as the stability of mRNAs, the effects of heavy metals on protease production, and post-translational modifications of laccase variants (Pezzella *et al*., 2009). To investigate how the two laccase genes are regulated by carbon and nitrogen sources, organic substances, copper, and osmotic stresses, we used mycelia collected at specific time point (day 9 of a liquid culture) and analyzed the transcriptional responses of the two laccase genes to different stimuli after a 6-h induction period. We also analyzed the transcriptional profiles of the two laccase genes during the degradation of aspen wafers, to determine which gene was responsible for the degradation of an intact woody substrate.

Materials and Methods

Enzymes, kits, chemicals, and media

All chemicals were analytical grade. Nucleic acid extraction kits were purchased from Tiangen Biotech Co., Ltd. Malt extract, yeast extract, and potato dextrose agar were obtained from BD or Oxoid. All enzyme substrates and aromatic substances required for analyses of laccase activities or to induce laccase expression were obtained from Sigma-Aldrich.

Organism and culture conditions

The brown rot fungus *P. placenta* strain MAD-698-R was kindly provided by Hammel KE (USDA Forest Mycology Center, USA). This strain was maintained at room temperature on malt extract and yeast extract agar (MY) plates (Wei *et al*., 2010). We tested the following media to determine their suitability as the basal medium for induction analyses: potato dextrose broth (PDB), malt extract glucose medium (MGY) (malt extract 15 g, 134 yeast extract 2 g, glucose 10 g, agar 20 g per L), MY medium, and low carbon and nitrogen medium (LNC) prepared with Highley's basal salt medium (Highley, 1973). Several studies have shown that the transcription of laccase genes was regulated differently during growth in white rot fungi (Soden and Dobson, 2001; Piscitelli *et al*., 2011). Therefore, we first evaluated the transcriptional profiles of *Pplcc1* and *Pplcc2* at different stages so that we

could accurately measure the changes in expression levels under different induction conditions. The dry weight of mycelia in submerged culture was determined according as described previously to obtain the growth curve for *P. placenta* in LNC medium (Manubens *et al*., 2007).

Inoculation, induction, and cultivation of fungal mycelia

To investigate the effects of different media on laccase gene expression, mycelia collected on day 9 of culture in LNC medium were disrupted using a Waring blender at maximum speed for 30 sec, and then collected by centrifugation (15,000 × g, 5 min). The pellets were washed twice with sterile distilled water. Then, the pellets (100 ± 10 mg) were inoculated into 20 ml liquid medium in 100-ml flasks. The cultures were incubated at room temperature (22–25°C) with agitation at 150 r/min for 12 days. The laccase activity in the supernatant of the liquid culture was determined daily as described previously (Wei *et al*., 2010).

To investigate the effects of carbon and nitrogen sources on the transcription of laccase genes, washed pellets (100 ± 10 mg, day 9 of liquid culture) were dispersed into Highley's basal salt medium without carbon and nitrogen as the control cultures, or into Highley's basal salt supplemented with glucose and ammonium tartrate. Stock solutions of glucose and ammonium tartrate were added to the basal salt medium to obtain the different concentrations (w/v: 0.5%, 1%, and 2% glucose, and 0.5% and 1% ammonium tartrate). After induction for 6 h, the mycelia were collected and used for RNA extraction.

To investigate the effects of copper on the transcription of laccase genes, washed pellets (100 ± 10 mg, day 9 of liquid culture) were inoculated into 20 ml fresh LNC medium. Then, 6 h before analyses, different volumes of a copper stock solution were added to the medium to induce laccase gene expression. The final concentrations of copper in the media were 50 μM, 200 μM, and 400 μM. LNC medium without copper was used as the control.

To investigate the effects of various aromatic substances on laccase gene expression, a filter-sterilized stock solution of each substance was added to the culture (9 days) in LNC medium, and laccase expression was evaluated after 6 h. The final concentration of each substance was 200 μM, because high concentrations of organic substances could negatively affect fungal growth (Vanhulle *et al*., 2007). Five different organic substances were evaluated: ferulic acid (FA), veratric acid (VA), 2, 6-dimethylbenzoic acid (DMBA), veratryl alcohol (VAL), and dimethoxyphenol (DMP). Each substance was dissolved in absolute ethanol to make a stock solution. Cultures supplemented with an equal amount of ethanol (0.2% v/v) were used as a control. Cultures with equal amount of water were used to investigate the effects of ethanol on the expression of laccase genes.

To explore the roles of the two laccases in this brown rot fungus, we monitored the expression of both genes under osmotic stresses imposed by mannitol, ammonium sulfate, and NaCl. In these experiments, 100 mg mycelia (day 9 of liquid culture) was added to fresh LNC medium containing the osmotic substance (at 0.2 M) and then gene expression was analyzed after 6 h. To investigate the effects of different concentrations of an osmotic substance on gene transcription, two concentrations of mannitol (0.2 M and 0.6 M) were used to impose osmotic stress. LNC medium with no osmotic substances was used as the control.

We investigated wood degradation by *P. placenta* using solid plates of different media (9 cm diameter) covered by mycelia. Aspen wood wafers (*Populus* sp.) with approximately the same size and weight (length × width × thickness = 10 mm × 5 mm × 2 mm, 150 ± 15 mg) were placed on top of the mycelial mat to allow the fungus to colonize the woody substrate. Two layers of nylon nets were placed between the wafers and agar to prevent contamination of the wafers by the medium. To determine which medium would be suitable to support the colonization and degradation of aspen wafers, we evaluated the degradation profiles of woody substrates on solid plates of four kinds of media for 8 weeks. Mycelia that were not in contact with the wafers were collected from the plate as the control. To monitor the degradation process, the weight and mechanical strength losses were recorded as described previously (Wei *et al*., 2010). All experiments were performed in triplicate.

RNA preparation and reverse transcription

Total RNA was extracted from submerged cultures using a modified mini-bead beater method (Leite *et al*., 2012). Total RNA from the mycelia inside the wafers at different stages of degradation was extracted as described previously (Wei *et al*., 2010). First-strand complementary DNA (cDNA) was synthesized with a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa) in a 20-μl reaction system with RT primer Mix and 1 μg total RNA according to the manufacturer's instructions. After dilution with EASY dilution buffer (1:1), 1 μl of the reaction mixture was used in the qPCR reaction.

Quantification of laccase gene expression under different conditions

The gene-specific primers used for qPCR analysis were designed with Beacon designer software (version 7). The primer sequences are listed in Table 1. Gene transcripts were quantified by qPCR using SYBR Green Supermix with ROX reference dye (Tiangen) on a Mastercycler EP Realplex system (Eppendorf) according to the manufacturer's instructions. Negative controls without cDNA were run under the same conditions to detect any contamination. Each PCR mixture contained 300 nM each primer, 1 μl diluted cDNA, and 10 μl SYBR Green Supermix in a final volume of 20 μl. Samples were amplified in 96-well plates. The amplification conditions were as follows: 15 min at 95°C, and then

Table 1. Primers used for real-time PCR in this study. Protein ID refers to annotation of *P. placenta* genome (http://genome.jgi-psf.org/Pospl1/Pospl1.home.html)

Protein ID	Primer name	Primer sequence (5′→3′)
XP_002471911.1	Tubulin F	5′-ACTCCGCATTTATTGGCTGGTT-3′
XP_002471911.1	Tubulin R	5′-CCGCATCGTCGAGATTACCG-3′
111314	111314 F	5′-GCCTTGCCTGGCATTGATTC-3′
11314	111314 R	5′-GCGTGGTCTGGTTGGTTTCG-3′
62097	62097 F	5′-TGTATGATGTTGATGATGATAGC-3′
62097	62097 R	5′-GGAATGGAGACCTGGATG-3′

40 cycles of 15 sec at 95°C, 20 sec at 60°C, and 30 sec at 72°C. The specificity of the amplification reactions was confirmed by constructing dissociation curves with a temperature range from 60°C to 95°C. Three biological replicates were included for each sample, and each replicate was analyzed three times. For all samples, the CT value for each target gene was normalized to the CT value of the reference gene. The amplification data were analyzed with the $^{\triangle\triangle}C_T$ method (Livak and Schmittgen, 2001). For the induction treatments, the results were normalized against the amplification of β-tubulin (endogenous control) cDNA on the same plate under the same conditions ($\Delta C_T = C_{T\ lcc} - C_{T\ tubulin}$). The relative amount of each target gene was calculated as an individual data point normalized with respect to β-tubulin and given as $2^{-\triangle CT}$ (Pezzella *et al.*, 2013). Values are the average of three replicates and error bars represent the standard deviation.

Results

Time course of laccase activity in four different liquid mediums

Laccase activity was repressed in PDB, and only one peak was detected on day 10. Two peaks of laccase activity were detected in the other media; the first peak on day 6 or 7, followed by minimum activity on day 8; and the second peak on day 9 (in LNC) or day 10 (in MY and MGY). After that, laccase activity decreased to a negligible level on day 12 in all media. The highest laccase activity was in LNC medium containing 1% glucose and 0.1% ammonium tartrate on day 9. This medium was selected as the basal medium to investigate gene induction and time course expression, because laccase activity was detected more easily in this medium than in the other media (Fig. 1).

Time course of laccase gene transcription at different growth phases in LNC medium

First, we obtained the growth curve for *P. placenta* in LNC. The lag phase was from day 1 to day 6, the exponential phase from day 6 to day 9, and the stationary phase from day 10 to day 12 (Fig. 2). On day 12, the mycelia became autolytic.

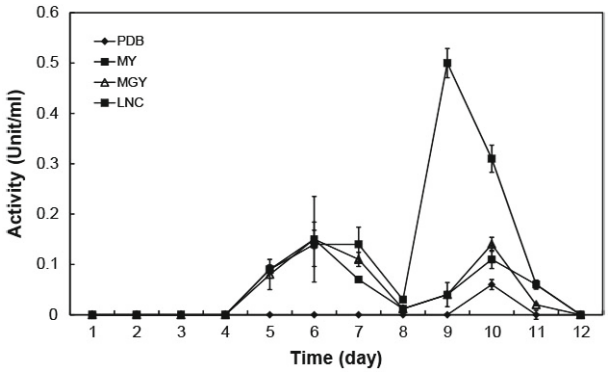

Fig. 1. Time course of extracellular laccase activity detected in PDB, MY, MGY, and LNC media. Values are mean ± standard deviation (n = 3).

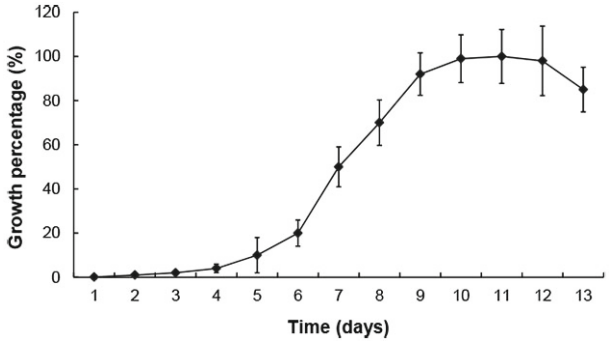

Fig. 2. Growth curve of *P. placenta* in submerged culture in LNC medium. Maximum weight in stationary phase was set to 100%. Values are mean ± standard deviation (n = 3).

Transcripts of *Pplcc1* and *Pplcc2* were detectable on day 2 (Fig. 3). The first peak in *Pplcc1* transcription was on day 4 and the second peak was on day 9. The transcript level of *Pplcc1* on day 9 was about 20-fold higher than that on day 4. The minimum transcript level of *Pplcc1* was on day 10, but a third peak in transcription, to a level similar to that on day 4, occurred on day 12. The transcript level of *Pplcc2* also increased from day 2 and peaked only once on day 6. After that, it declined to about approximately half the level on day 6 on days 8 and 9. Transcription of *Pplcc2* decreased to a negligible level after day 10. Therefore, transcription of the two genes was differentially regulated at different growth stages, and neither of them was expressed constitutively. The highest transcript level of *Pplcc1* was at the end of the linear stage in the growth curve, and the highest transcript level of *Pplcc2* was during the exponential growth phase (Fig. 3). We selected mycelia from day 9 of liquid culture to detect transcriptional responses because both genes were transcribed at relatively high levels at this time, making it easier to detect changes in their transcript levels in response to added compounds.

Transcriptional responses of *Pplcc1* and *Pplcc2* to changes in carbon and nitrogen concentrations

In medium containing 0.5% ammonium tartrate, the trans-

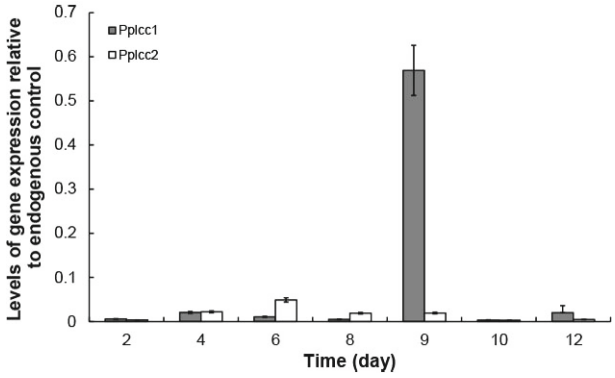

Fig. 3. Time course of *Pplcc1* and *Pplcc2* transcription in *P. placenta* in LNC medium at different growth stages. Values are mean ± standard deviation (n = 3).

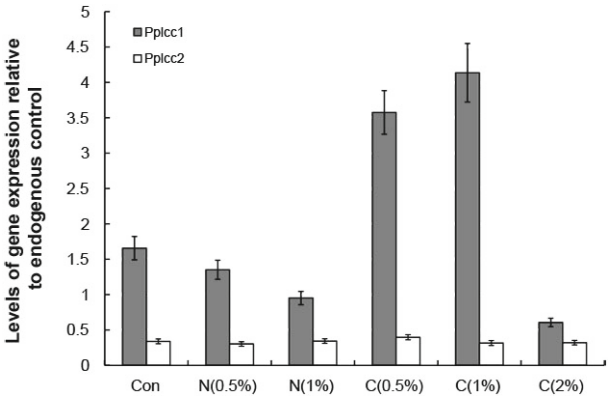

Fig. 4. Transcriptional regulation of *Pplcc1* and *Pplcc2* by different concentrations of glucose and ammonium tartrate. Con, control culture with no carbon and nitrogen sources; N, nitrogen; C, carbon. Values are mean ± standard deviation (n = 3).

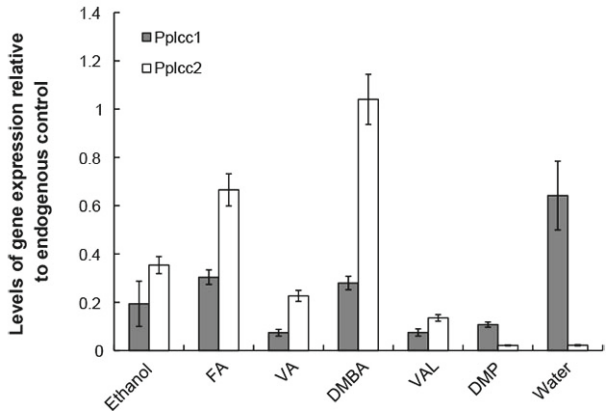

Fig. 6. Transcriptional regulation of *Pplcc1* and *Pplcc2* by organic substances. FA, ferulic acid; DMBA, 2, 6-Dimethylbenzoic acid; VA, veratric acid; VAL, veratryl alcohol. Values are mean ± standard deviation (n = 3).

cript level of *Pplcc1* was four-fifths that in the control. Increasing the ammonium tartrate concentration to 1% further repressed the transcription of *Pplcc1* to about three-fifths that in the control. However, the transcript levels of *Pplcc2* did not change in response to nitrogen enrichment. Nitrogen depletion upregulated the transcription of *Pplcc1* but not *Pplcc2* (Fig. 4).

The transcript level of *Pplcc1* in basal medium with 0.5% glucose was 2-fold higher than that in the control. Increasing the glucose concentration to 1% further stimulated *Pplcc1* transcription to a level about 2.6-fold higher than that in the control. However, in medium containing 2% glucose, the transcription of *Pplcc1* was significantly inhibited to a level approximately one-third that in the control. The transcription of *Pplcc2* was slightly upregulated when glucose was included in the medium, but it remained at almost constant levels when the glucose concentration was further increased.

Transcriptional responses of *Pplcc1* and *Pplcc2* to different chemical stimuli

The transcription of *Pplcc1* was gradually upregulated as the copper concentration increased from 50 μM to 400 μM. The transcript levels of *Pplcc1* in media containing 50 μM, 200 μM, and 400 μM copper were approximately 1.4-, 6-, and 9-fold higher, respectively, than that in the control. The transcription of *Pplcc2* was only slightly upregulated by 50 μM copper, to a level approximately 1.5-fold higher than that in the control. Further increases in the copper concentration did not affect *Pplcc2* transcription (Fig. 5).

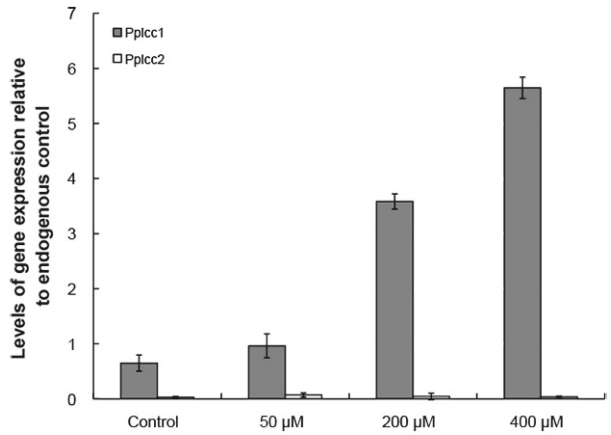

Fig. 5. Transcriptional regulation of *Pplcc1* and *Pplcc2* by different copper concentrations. Values are mean ± standard deviation (n = 3).

The inclusion of ethanol (0.2%) alone decreased the transcription of *Pplcc1* to one-third of that in the control supplemented with water (Fig. 6). However, the transcription of *Pplcc2* was upregulated by ethanol. The transcript level of *Pplcc2* in medium containing 0.2% ethanol was approximately 15-fold higher than that in medium supplemented with water. The relative transcript level of *Pplcc1* were much higher (approx. 30-fold higher) than that of *Pplcc2* in the culture supplemented with water. However, in medium containing ethanol, the transcript level of *Pplcc2* was approximately 1.5-fold higher than that of *Pplcc1*. When phenolic compounds were included in the culture medium with ethanol, only FA and DMBA upregulated the transcription of both genes. The transcript levels of *Pplcc1* in medium containing FA and in medium containing DMBA were about 1.5-fold higher than that in medium containing only ethanol. The transcript levels of *Pplcc2* in medium containing FA and in medium containing DMBA were about 2-fold and 4-fold higher, respectively, than that in medium containing only ethanol (Fig. 6).

Transcriptional responses of *Pplcc1* and *Pplcc2* to osmotic stresses

When different substances (at a final concentration of 0.2 M) altered the osmotic potential of LNC medium, *Pplcc2* became the major transcript. The relative expression level of *Pplcc2* was at least 2-fold higher than that of *Pplcc1* under osmotic stress imposed by mannitol, NaCl, and ammonium sulfate (Fig. 7A). In the control (LNC), *Pplcc1* was always the major transcript (Fig. 5). This result indicated that osmotic stress reversed the expression profiles of the two genes. In media containing 0.2 M and 0.6 M mannitol, the higher

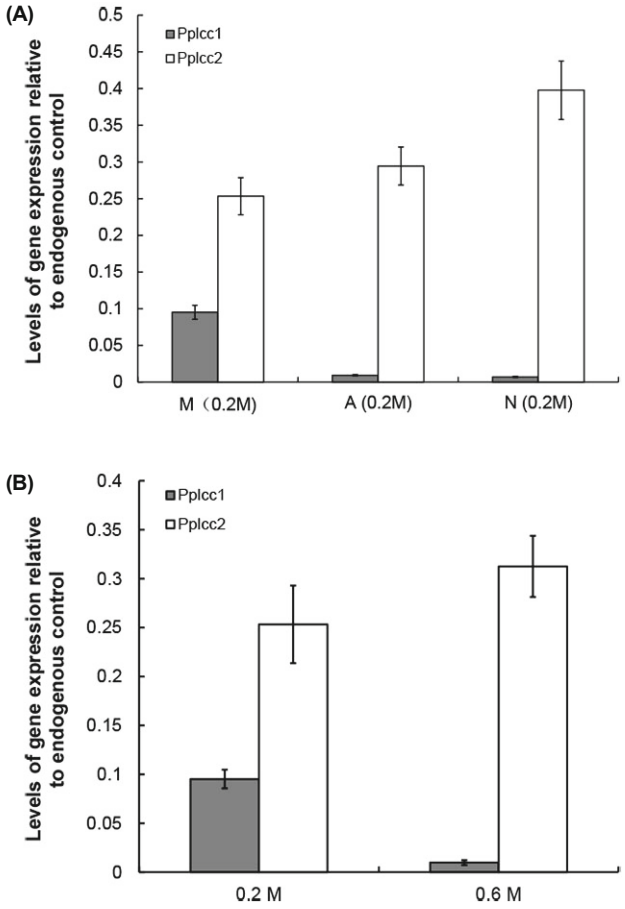

Fig. 7. Transcriptional regulation of *Pplcc1* and *Pplcc2* by osmotic stress. (A) Relative transcript levels of *Pplcc1* and *Pplcc2* in LNC medium with different substrates (0.2 M). M, mannitol; A, ammonium sulfate; N, NaCl. (B) Effect of mannitol concentration (0.2 M and 0.6 M) on transcription of *Pplcc1* and *Pplcc2*. Values are mean ± standard deviation (n = 3).

concentration further upregulated the transcription of *Pplcc2* and downregulated that of *Pplcc1* (Fig. 7B). When the concentration of mannitol increased from 0.2 M to 0.6 M, the transcript level of *Pplcc2* was about 15-fold higher than that of *Pplcc1*. These results indicated that *Pplcc2* might be responsible for adaption to osmotic stress.

Effects of different solid media on colonization and degradation of aspen wood wafers

The degradation process did not differ substantially among the MY, MGY, and LNC plates, but there were significant differences in degradation on PDA medium. The degradation process was monitored over 8 weeks and the characteristics were typical for brown rot. On the MY, MGY, and LNC plates, the aspen wafers showed a 4–6% weight loss, with significant stiffness loss (60–70%) at week 3. On the PDA medium, there was a 2% weight loss with a 10% stiffness loss at week 3. Therefore, the degradation process was inhibited on PDA plates, just like laccase activity was repressed in liquid PDB medium (Fig. 8). However, the stiffness loss was still about 80% by the end of the 8-week degradation process on PDA medium. These results showed

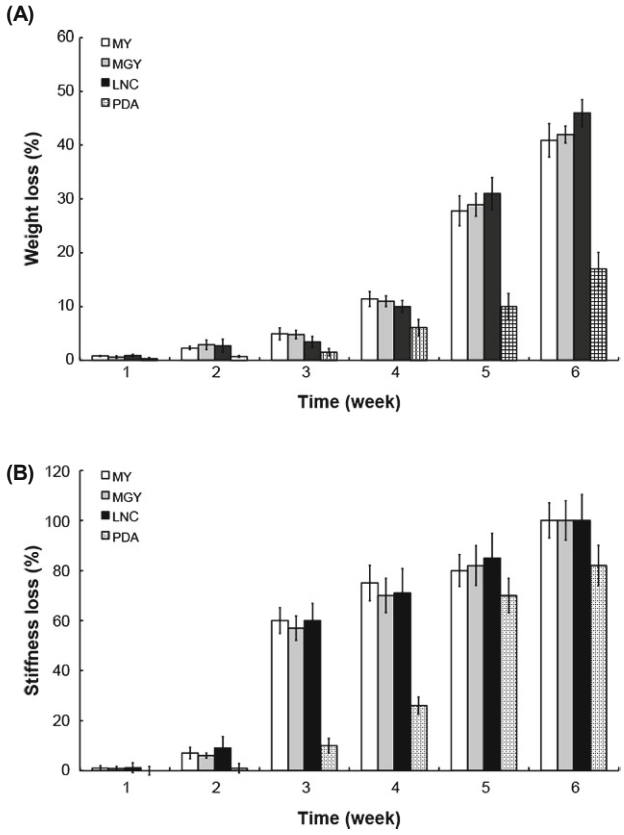

Fig. 8. Degradation profiles of aspen wafers by *P. placenta* on plates of four different media. (A) Percentage weight loss of aspen wafers. (B) Percentage stiffness loss of aspen wafers. Values are mean ± standard deviation (n = 3).

that all four media could support the degradation of aspen wafers, but PDA was not an ideal substrate to support wood decay.

Time course expression of *Pplcc1* and *Pplcc2* during degradation of aspen wafers

The transcript levels of *Pplcc1* in aspen wafers increased gradually on day 3 and day 10. The highest expression level

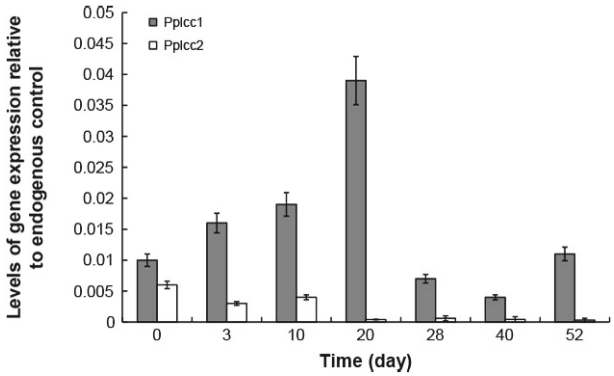

Fig. 9. Relative transcript levels of *Pplcc1* and *Pplcc2* during degradation of aspen wafers on MY plate. Values are mean ± standard deviation (n = 3).

of *Pplcc1* (approximately 4-fold higher than that on day 0) was on day 20. After that, the *Pplcc1* transcript levels decreased to approximately one-quarter that in the control (day 0) by day 40. Interestingly, *Pplcc1* showed a second peak in transcription on day 52, when its transcript level was approximately the same as that in the control (day 0) (Fig. 9). Therefore, the transcription of *Pplcc1* was stimulated by contact with the woody substrate during the initial colonization phase. However, the transcription of *Pplcc2* was inhibited by contact with the aspen wafer, with transcript levels decreasing to approximately half of that in the control (day 0) by day 3. By day 10, the transcript level of *Pplcc2* had increased to a level approximately 1.2-fold higher than that on day 3. Then, it decreased to its minimum level at day 20 (one-twentieth of the level in the control; day 0). In intact aspen wafers, the relative transcript level of *Pplacc2* was generally lower than that of *Pplcc1* during the whole degradation process (Fig. 9).

Discussion

For many years, brown rot fungi were thought to lack laccases because they could not be detected using traditional detection methods. Therefore, the physiological roles of laccases in brown rot fungi have rarely been investigated. However, with increasing evidence for the existence of laccases in brown rot fungi, more efforts should be made to unravel their physiological roles.

In white rot fungi, the expression of some laccase isoforms in the lag and exponential phases is related to substrate degradation, while the expression of other isoforms in the stationary phase is related to morphogenesis and pigmentation (Lettera *et al.*, 2010). In *P. placenta*, *Pplcc2* appeared to be involved in substrate degradation, because its transcription peaked on day 6, during the lag phase. On day 4, the relative transcript level of *Pplcc1* was comparable to that of *Pplcc2*, but *Pplcc2* was the major transcript during the lag phase (Fig. 3). The highest transcript level of *Pplcc1* was on day 9, at the end of the exponential phase. *Pplcc1* was also involved in the degradation of woody substrates-the high transcript level of *Pplcc1* on day 9 may reflect its response to low concentrations of nitrogen and/or carbon sources, similar to the situation when the mycelia first colonize wood. The third peak of *Pplcc1* transcription was on day 12, during the stationary phase. However, there was no apparent pigment formation or special morphogenesis during the whole process (data not shown). Therefore, the results obtained in this investigation do not support a role for *Pplcc1* in morphogenesis and pigmentation.

The expression of laccase genes in some ligninolytic basidiomycetes was shown to be upregulated under both non-limiting and limiting nitrogen conditions (Piscitelli *et al.*, 2011). In *P. placenta*, the expression of *Pplcc2* was not affected by changes in nitrogen concentrations. The relative transcript level of *Pplcc2* (compared with that of the endogenous control) was approximately 0.35 in all of the treatments and in the control. Therefore, *Pplcc2* expression was not responsive to non-limiting and limiting nitrogen conditions. However, the relative expression level of *Pplcc1* increased from 0.9 to 1.7 when the concentration of ammonium tartrate decreased from 1% to 0% (Fig. 4). Therefore, *Pplcc1* was upregulated under nitrogen-limited conditions. The response of *Pplcc1* to nitrogen starvation was similar to that of fungal peroxidases (Li *et al.*, 1994). The different responses of *Pplcc1* and *Pplcc2* to nitrogen depletion may be partly explained by differences in their promoter sequences; *Pplcc1* has five putative NIT2 (nitrogen regulatory protein) DNA-binding motifs (consensus sequence: TATCDH) (Fu *et al.*, 1990) in its promoter sequence, while *Pplcc2* has only two of these motifs. The NIT2 binding site has been shown to mediate the transcription of laccase genes under low-nitrogen conditions (Fu *et al.*, 1997).

In some cases, increasing the concentration of easily metabolized carbon sources such as glucose can lead to higher laccase activity than that achieved with other carbon sources (Periasamy and Palvannan, 2010). A very high concentration of glucose (15 g/L) inhibited laccase expression in the basidiomycetes CECT 20197, *Trametes pubescens*, and *Trametes* sp. AH28-2 (Mansur *et al.*, 1997; Galhaup *et al.*, 2002; Xiao *et al.*, 2006). In this study, increasing glucose concentrations upregulated *Pplcc1* transcription to some extent, but did not affect the transcription of *Pplcc2*. Catabolite repression was observed for *Pplcc1* when the concentration of glucose increased to 2%. Therefore, a dose-dependent repression mechanism might exist in *P. placenta*. The expression of *Pplcc1* was induced during glucose starvation and repressed when excess sugars were present. This mechanism may be important for the role of laccase in the decay process. The presence of putative consensus sequence motifs such as the CreA binding site (consensus sequence: SYGGRG) in the promoter region of laccase genes has been suggested to be involved in their repression by catabolites (Strauss *et al.*, 1999). Four putative CreA motifs were detected in the promoter region of *Pplcc1* but only one was detected in the promoter region of *Pplcc2*.

The different responses of the two laccase genes to copper could be explained by the presence of two kinds of motifs in their promoters; the metal responsive element (MRE), and the activation of CUP1 expression (ACE) binding site (Thiele, 1992). Five putative MRE motifs were detected in the promoters of both genes, but an additional four ACE motifs were present in the promoter of *Pplcc1*. No ACE motifs were detected in the *Pplcc2* promoter. There are three possible explanations for different responses of the two genes. First, although both promoters contained five MREs, individual MRE elements vary in their metal-activated transcriptional potency (Thorvaldsen *et al.*, 1993). Second, in most cases, the transcriptional activation of target genes by MREs requires a Zn-dependent binding factor (Thiele, 1992), but no additional Zn was included in these experiments. Third, the ACE-binding site is the target-specific sequence for the ACE1 copper-responsive transcription factor originally found in *Saccharomyces cerevisiae*. The induction of a laccase gene by copper in the white rot fungus *Ceriporiopsis subvermispora* was shown to be mediated by an ACE1-like copper-fist transcription factor that specifically interacts with the ACE element in the laccase gene promoter (Manubens *et al.*, 2007). In *P. chrysosporium*, ACE elements in the promoter region of *MCO* genes have been shown to mediate their induction

by copper. Only *mco1*, which contains ACE elements in its promoter, could be upregulated by copper (Canessa *et al*., 2008). Our results were consistent with these reports. Therefore, the response of *Pplcc1* to copper is likely to be mediated by the ACE pathway in *P. placenta*.

In some fungi, ethanol was shown to increase the production of laccases by increasing membrane permeability and promoting protein secretion (Meza *et al*., 2007). Ethanol was also shown to increase gene expression and inhibit protease activity, which contributed to increase laccase production in the *Pycnoporus cinnabarinus* ss3 strain (Lomascolo *et al*., 2003). A northern blot analysis confirmed that ethanol promoted the transcription of *Lac1* in the *P. cinnabarinus* ss3 strain (Lomascolo *et al*., 2003). Our results indicated that the two laccase isoforms in *P. placenta* responded differently to ethanol induction. Ethanol is a stress factor that can indirectly induce laccase synthesis via oxidative stress caused by membrane disruption, using calcium as secondary messenger (Alves *et al*., 2004). The different responses of the two *P. placenta* laccase isoforms to ethanol could be because of differences in their promoter regions. Two putative stress responsive element (STRE) motifs (Mager and De Kruijff, 1995) were detected in the promoter of *Pplcc2*, but none were detected in the promoter of *Pplcc1*.

The different responses of laccase isoforms to aromatic compounds have been extensively investigated in white rot fungi, in which certain laccases are specifically induced by certain aromatic compounds (Piscitelli *et al*., 2011). Our results were not totally consistent with this rule. The transcriptions of both *Pplcc1* and *Pplcc2* were induced by FA and DMBA but inhibited by VA, VAL, and DMP. The induction of both genes by FA and DMBA could be because both contain a putative XRE motif (consensus sequence: CACGCW) in their promoters (Rushmore *et al*., 1990). The expression of *Pplcc2* was more sensitive than that of *Pplcc1* to aromatic substances, possibly because *Pplcc2* has two STRE motifs in its promoter. Laccase isoforms have been suggested to protect the cells against toxic compounds by reducing oxidative stress caused by oxygen radicals derived from reactions with aromatic compounds (Youn *et al*., 1995). This result also implies that the role of Pplcc2 might be to oxidize aromatic compounds.

In some white rot fungi, laccase or manganese peroxidases are induced under osmotic stress. For example, when *Lentinula edodes* mycelia were immersed in water for 24 h, the transcript level of one laccase gene decreased over time (Ohga and Royse, 2001). When the white rot fungus *Phlebia* sp. strain MG-60 was subjected to hypersaline conditions (2% NaCl), the transcription of two manganese peroxidase (MnPs) genes, *mnp2* and *mnp3*, was induced during the 48-h treatment (Kamei *et al*., 2008). In this study, *Pplcc1* and *Pplcc2* also responded differently to osmotic stress. The expression of *Pplcc1* was inhibited and that of *Pplcc2* was induced. This response could be related to the presence of two stress response elements (consensus sequence: CCCCT) in the promoter region of *Pplcc2*.

The time course of laccase expression during the degradation of intact wood has never been investigated, even for white rot fungi. However, the expression of other genes that could be related to wood degradation has been investigated during the degradation of modified wood. Another study showed that the transcript levels of genes encoding quinone oxidoreductase (Protein ID 124517) and alcohol oxidase (Protein ID 118723) increased at the start of colonization of pine wafers, and peaked on day 10 in *P. placenta* (Ringman *et al*., 2014). The increased activity of quinone oxidoreductase provided sufficient hydroquinones by reducing 5-dimethoxycatechol or 2, 5-dimethoxyhydroquinone (Paszczynski *et al*., 1999). Therefore, sufficient semiquinone radicals could be produced to reduce Fe^{3+} to Fe^{2+}. The increased expression of alcohol oxidase provided another source of H_2O_2 for Fenton reaction. Our results indicate that the contact with aspen wafers stimulated the expression of *Pplcc1,* which is thought to be involved in the colonization of a woody substrate. Together, these results show that the transcript levels of several genes supporting the hydroquinone-driven Fenton reaction increased during the initial colonization of aspen wafers.

The expression level of *Pplcc2* decreased when the mycelia came into contact with wafers. This result was unexpected, considering that expression of *Pplcc2* was induced by FA and DMBA in liquid culture (Fig. 6). One reason for this contradiction might be that different aromatic compounds selectively regulated the production of the distinct laccase isoforms, as demonstrated in some fungi (Piscitelli *et al*., 2011). In *P. placenta*, other aromatic compounds such as VA, VAL, and DMP downregulated the expression of *Pplcc2* (Fig. 6). Therefore, the aromatic components in lignin could affect the final expression level of *Pplcc2*.

In conclusion, this is the first systematic study on the transcriptional regulation of laccases in brown rot fungi. These results indicate that *Pplcc1* is more responsive than *Pplcc2* to changes in carbon, nitrogen, and copper concentrations in liquid culture. *Pplcc2* positively responded to several stresses in liquid culture. When the mycelia came into contact with intact aspen wafers, *Pplcc1* expression responded positively to the woody substrate, and became the major transcript during degradation. Considering that peptides of only Pplcc1 were detected in degraded wafers in our previous study, this isoform is likely to be the main contributor to the Fenton reaction by oxidizing hydroquinone, followed by the reduction of Fe^{3+} to Fe^{2+} in *P. placenta* (Wei *et al*., 2010). For *Pplcc2*, the downregulated transcription upon contact with wafers indicated that some aromatic compounds in the wood might inhibit its expression. However, the much higher expression level during the initial colonization stage than at the later stage of degradation suggested that it plays a role in colonizing the woody substrate.

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