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Molecular Characteristics of Two Laccase from the Basidiomycete Fungus *Polyporus brumalis*

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Two laccase cDNAs, *pblac1* and *pblac2*, were cloned from a white-rot fungus strain, *Polyporus brumalis* (KFRI 20912). The cloned cDNAs consisted of 1,829 bp and 1,804 bp, and their open reading frames encoded proteins of 520 and 524 amino acids, with calculated molecular masses of approximately 55.9 kDa and 56 kDa, respectively. The deduced amino acid sequences of each protein showed 70% similarity. The copper binding regions were conserved in both proteins, as in other fungal laccases. RT-PCR analysis revealed that the transcript levels of the two laccases increased progressively in shallow stationary culture liquid medium. The transcript level of each laccase was induced when the fungus was exposed to di-butyl phthalate (DBP), suggesting that the two laccases are involved in DBP degradation. The overexpression of the *pblac1* gene was derived by the promoter of a gene for glyceraldehyde-3-phosphate dehydrogenase, using a homologous system. The activity of laccase in the transformants was significantly higher than that of the wild type. The identification of these laccase cDNAs was a first step to characterize the molecular events related to the lignin degradation ability of this basidiomycetous fungus, as well as the degradation of many recalcitrant xenobiotics.

***Keywords*:** laccase cDNA, white rot fungi, *Polyporus brumalis*

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to the copper-containing oxidases. It was first found in the varnish tree *Rhus vernicifera*; however, most laccases have been found and studied in lignin-degrading basidiomycetes, white rot fungi (Yoshida, 1883; Reinhammar, 1984; Thurston, 1994). The ability of white rot fungi to degrade lignin and many aromatic xenobiotics is related to this enzyme (Xu, 1996; Mayer and Staples, 2002). Laccase catalyzes the oxidation of phenolic compounds and aromatic amines with molecular oxygen as the electron acceptor (Palmieri *et al.,* 1993), because its oxidation potential would not be high enough to enable it to oxidize nonphenolic structures. However, recent work by Eggert *et al.* (1996) has identified a fungal metabolite of *Pycnoporus cinnabarinus* that mediates nonphenolic lignin degradation by laccase. There are many reports about the potential application of laccase in pulping, textile dyes, biosensors, and the detoxification of polluted water (Palmieri *et al.,* 1993; Reid and Paice, 1994; Martirani *et al.,* 1996; Van Aken *et al.,* 1997; Han *et al.,* 2004).

Furthermore, genes encoding for laccase are required for these functions, and for efficient heterologous expression systems (Berka *et al.,* 1997; Bailey *et al.,* 2004). So far, cDNA and laccase genes have been isolated by molecular cloning from some white rot fungi such as *Trametes versicolor* (Ong *et al.,* 1997; Cassland and Jonsson, 1999; Cheong *et al.,* 2006), *Ceriporiopsis subvermispora* (Karahanian *et al.,* 1998), *Lentinula edodes* (Zhao and Kwan, 1999; Ohga and Royes, 2001), and *Coprinus congregatus* (Kim *et al.,* 2001). *Trametes* species are shown to degrade 2,4,6-trinitrotoluene (TNT), and laccase genes are frequently expressed during its degradation, suggesting that the laccase in these fungi is implicated in the degradation of TNT and its catabolites (Cheong *et al.,* 2006; Gibson *et al.,* 2006). However, the specific functions of these laccase genes during biodegradation are not well-known.

Polyporus brumalis causes white soft rots on dead hard wood and stumps, as well as on fallen branches and trunks. This fungus has shown resistance to di-butyl phthalate (DBP) treatment at a concentration of 250 μM, and its DBP degradation efficiency was approximately 95% after 12 days of incubation (Lee *et al.,* 2005). In addition, the oxidation of nonphenolic polycyclic aromatic hydrocarbons such as DBP has been observed by laccases from *P. brumalis*. Therefore, *P. brumalis* is expected to be a superior strain for DBP degradation.

In order to investigate the function of laccase and its regulatory mechanisms on the degradation of recalcitrant materials such as DBP, we cloned and determined the nucleotide sequences of laccase genes from *P. brumalis*. We then investigated the expression patterns in the fungus, and constructed transformants using a homologous system for the expression of the cloned laccase genes in *P. brumalis*.

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Materials and Methods

Fungal cultures and DBP treatment

The *P. brumalis* strain was maintained on potato dextrose agar (PDA) plates, and grown in 300 ml of liquid potato dextrose broth (PDB) or shallow stationary culture (SSC) liquid medium [dextrose 15 g, $(NH_4)_2C_4H_4O_6$ 1 g, thiamine-HCl 0.5 mg, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $Ca(H_2PO_4)_2$ 0.5 g, mineral solution 5 ml ($MgSO_4$ 3 g, NaCl 1 g, $MnSO_4$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.1 g, $CoCl_2$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, $CuSO_4$ 0.1 g, $AlK(SO_4)_2 \cdot 12H_2O$ 10 mg, nitriloacetic acid 1.5 g/distilled water 1,000 ml); and distilled water, 1,000 ml] at 28°C (Tien and Kirk, 1984). For the DBP treatments, *P. brumalis* was pre-grown in SSC liquid medium for 5 days at 28°C, and treated with 100 and 300 μM DBP, and then incubated for 9 and 15 days.

Laccase activity assays

Laccase activity was determined by the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate. The culture broth was clarified by centrifugation at 10,000 rpm for 10 min. The reaction mixture was contained in 0.2 M lactate buffer (pH 3.0). The ABTS oxidation was followed by an absorbance increase at 414 nm. The enzyme activity was expressed in units defined as the amount of enzyme oxidizing 1 μmol of ABTS per min (ε=36,000/M/cm). The kinetic studies were performed by measuring the initial velocity and the velocities of the enzyme-catalyzed reactions measured at 414 nm for ABTS. The protein assay was determined by the Bradford method (Bio-Rad, USA).

Cloning of laccase genes by RT-PCR

The total RNA was extracted with Trizol reagent (Invitrogen, USA) from *P. brumalis* mycelia. The amount and quality of the RNA were determined by absorbance at 260 and 260/280 nm, respectively. The first cDNAs were synthesized by transcribing 1 μg of RNA with 200 units of MMLV reverse transcriptase (Promega, USA), as recommended by the manufacturer. Approximately 1 kb fragments were amplified with pairs of degenerated oligonucleotide primers; 5'-CAYTGG CAYGGNTTYTTYCA-3' and 5'-GHWMBTHYTGGTRBC ACWSYC-3'. PCR was performed as follows: initial denaturing at 94℃ for 120 sec, 30 cycles of denaturing at 94°C for 60 sec, annealing at 55°C for 30 sec, elongation at 72°C for 90 sec, and ending with a 10 min final extension at 72℃. The amplified fragments were subcloned into a pCR2.1 TOPO (Invitrogen) vector, and sequencing of the double-stranded plasmid DNA was performed.

Rapid amplification of cDNA ends (RACE)

We performed RT reactions from 1 μg of purified total RNA with the Smart RACE cDNA Amplification kit (Clontech, USA), in order to amplify the 5'- and 3'-cDNA ends of the *pblac1* and *pblac2* transcripts. The 5' and 3' RACE reactions were carried out according to the instructions of the manufacturer, using the outer gene specific primers: GSPs of *pblc1*; 5'-GGACCGAGCCTAGCAGCAACG-3', 5'-CGTCAT TACACTGGCAGATTGG-3', and *pblac2*; 5'-CTGACCGGC GTAGATCTGGATG-3', 5'-GACCATCGAGCTCTCCTTCC CG-3', and inner (nested) gene coding sequence specific primers: NGSPs of *pblac1*; 5'-CCAATCTGCCAGTGTAATGA CG-3', 5'-CGTTGCTGCTAGGCTCGGTCC-3' and *pblac2*; 5'-CATCGACAACCCCACCAAAAAAG-3', 5'-CACCCCTTCC ATCTGCACGG-3' that were designed for *pblac1* and *pblac2.* The PCR conditions were as follows: 5 cycles of denaturing at 94°C for 30 sec, annealing and elongation at 72°C for 180 sec, 5 cycles of denaturing at 94°C for 60 sec, annealing at 70°C for 30 sec, elongation at 72°C for 180 sec, 25 cycles of denaturing at 94°C for 60 sec, annealing at 68°C for 30 sec, and finally, elongation at 72°C for 180 sec. The next steps were performed as described for the RT-PCR. Then, the full-length cDNAs of *pblac1* and *pblac2* were amplified by RT-PCR, with 5' and 3' primers designed according to the nucleotide sequence data obtained from the subcloned and sequenced RACE-PCR products.

The sequences of the full length cDNAs have been deposited in GenBank under the accession numbers EF362634 (*pblac1*) and EF362635 (*pblac2*).

Determination of gene expression

An RT-PCR kit (Promega) was used to identify the mRNA coding for laccase, and an 18S rRNA served as the internal control. The reaction products (20 μl) were analyzed by gel electrophoresis. The gene specific primers for the PCRs were designed from the 3' UTRs of each gene. The *pblac1* primer set (5'-GTCACTATTAAACTCGGGTATT-3' and 5'-AG TCGCAATTTGCTTCG-3') generated a 199 bp product, and the *pblac2* primer set (5'-GCGGACTGCTGTATCTC-3' and 5'-ATACTTAAATCATCCTCGC-3') amplified a 120 bp product. The nucleotide sequences of the RT-PCR products were identical to those of the corresponding laccase cDNAs.

Analysis of DNA and protein sequences

The obtained nucleotide sequences and homologous sequences were detected using database searches with the BLAST search program of the NCBI web-server. To predict the molecular weights and signal peptides of the deduced protein we used the ExPasy program (http://www.expasy.org/tools/). A sequence alignment was constructed with the CLUSTAL X and Genedoc programs.

Southern blot analysis

The genomic DNA of the *P. brumalis* mycelia was digested completely with *Xho*I-, *Eco*RV-, *Bam*HI-, and *Pst*I, fractionated on 0.8% agarose gel, and blotted onto a nylon membrane (PerkinElmer Life Sciences). The membranes were probed with ^{32}P-labeled PCR products to the 3'-untranslated regions (197 bp and 123 bp) of the two laccase cDNAs and the partial coding region (557 bp) of *pblac1.* The hybridization was carried out at 42°C overnight, and then washed under high or low stringency conditions according to the method of Church and Gilbert (1984).

Transformation by REMI and laccase activity assay during culture

We constructed a pHYlac1 vector using the modified plasmid of pBARGPE1 as follows: the transforming vector was replaced with the hygromycin resistance gene (*hph*), instead of the phosphinothricin resistance gene (*bar*), for the selectable maker, which contained the open reading frame se-

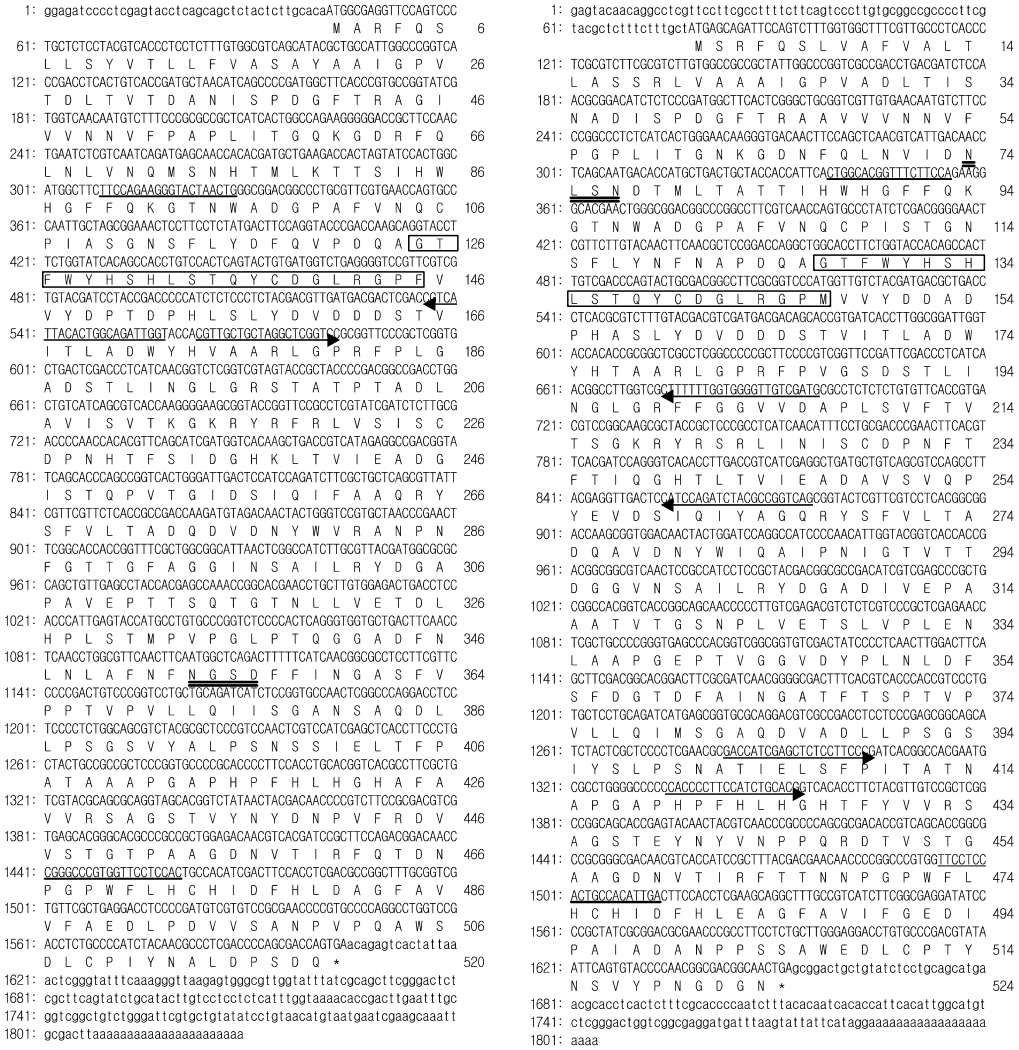

Fig. 1. DNA and deduced amino acid sequences of the laccase genes, *pblac1* (left) and *pblac2* (right). The primer sequences for the confirmation of laccase by RT-PCR are indicated by the underlines. Sequences with arrows represent the primers used for RACE. The possible N-glycosylation sites are marked by double lines, and the multi-copper oxidase signature sequences are indicated in boxes.

quences of the *pblac1* cDNA under the control of the gpd promoter. The transformation of *P. brumalis* was performed by the REMI method as described by Leem *et al*. (1999), with slight modifications. Protoplasts were generated using 0.5% USUKizyme. The transformants were selected from minimal medium containing 50 μg/ml hygromycin B.

The laccase activities of the transformants and wild type were analyzed by the oxidation of *o*-tolidine as a substrate. The aqueous solution of shallow stationary culture (SSC) liquid medium was used during 10 and 11 days of incubation at 25°C. Stock solutions of 4.7 mM *o*-tolidine were prepared in 95% ethanol with 67 mM glycine and 2 mM glacial acetic acid. The assays were carried out in 96-well multi-micro plates in a total volume of 300 ml at room temperature. The reaction was initiated by adding 75 μl of liquid culture medium, and then absorption spectra were taken after 5 min of incubation using a microplate spectrophotometer (Bio-Rad).

Results and Discussion

Cloning of laccase cDNA from *P. brumalis*

In general, laccase genes have 4 copper-binding regions that are highly conserved in various organisms. Therefore, the fragments flanked by these conserved sequences can be amplified by PCR using degenerated oligonucleotide primers. The degenerated primers are synthesized to be based on the conserved sequences. By using RT-PCR methods, we obtained 2 PCR products, 1,152 bp and 1,176 bp (referred to as *pblac1* and *pblac2*) (Fig. 1). When the fragment sequences were detected using database searches with the BLAST program of NCBI, they showed 75~92% homologies to known laccase genes.

Next, 5'-RACE and 3'-RACE were performed to sequence the N- and C-terminals, using gene specific primers based on the partial cDNA sequences obtained by RT-PCR. The RACE of *pblac1* yielded a 557 bp fragment of the 5'-region and a 1,267 bp fragment of the 3'-region; whereas *pblac2*

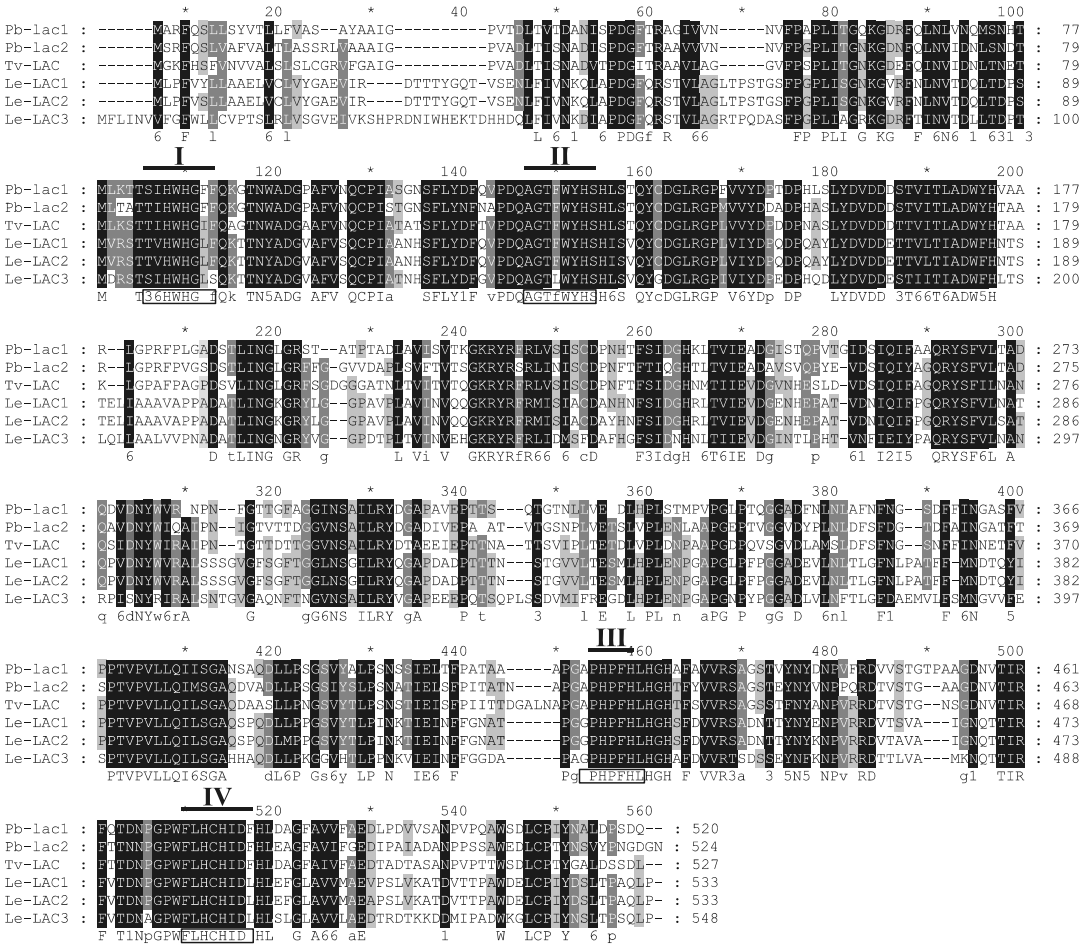

Fig. 2. Alignment of the deduced *pblac1* and *pblac2* amino acid sequences with those of other white rot fungi laccases. Four potential copper-binding domains are indicated by horizontal lines (I, II, III, and IV). The accession numbers of the sequences are as follows: Tv-LAC (*Trametes versicolor*), BAD98308; Le-LAC1 (*Letinula edodes*), AAT99287; Le-LAC2 (*Letinula edodes*), BAB83132; Le-LAC3 (*Letinula edodes*), AAT99291. The alignment was performed with CLUSTAL X1.81 software.

had a 696 bp fragment of the 5'-region and a 470 bp fragment of the 3'-region. These two 5'- and 3'-flanking regions were assembled to synthesize the full-length cDNA genes, respectively. The resulting dsDNA-products were amplified by RT-PCR with 5' and 3' primers designed according to the nucleotide sequence data that were obtained from RACE-PCR and then sequenced. The nucleotide sequences were deposited in GenBank under the accession numbers EF362634 (*pblac1*) and EF362635 (*pblac2*).

Sequence analysis of laccase genes

The nucleotide sequences of the cDNAs encoding laccase and their deduced amino acid sequences are shown in Fig. 1. Here, *pblac1* consisted of 520 amino acids with a molecular mass of approximately 55.9 kDa, and *pblac2* contained 524 amino acids with a molecular mass of approximately 56 kDa. Typically, fungal laccases are extracellular, glycosylated proteins of 60~85 kDa, of which 15~20% is carbohydrate (Thurston, 1994; Xu, 1999). The similarity between *pblac1* and *pblac2* is 70%, and both genes have putative N-glycosylated sites at the amino acid positions 354 and 74, which confers secretion outside cells without a C-terminal extension. Multi-copper oxidase signatures were present at amino acid positions 125-145 of *pblac1* (GtFwYhShLstqycDGLrgpF), and at 127-147 of *pblac2* (GtFwYhShLstqycDGLrgpM), which are contained in many of the proteins in the multi-copper oxidase family (Nakamura and Go, 2005).

Laccase can oxidize phenolic compounds, thereby creating phenoxy radicals, while nonphenolic compounds are oxidized via cation radicals. Laccases oxidize aromatic compounds with relatively low redox potentials, whereas compounds with higher ionization potentials are readily oxidized by LiPs (De Jung *et al.,* 1994). Laccases are divided into three classes based on an amino acid that is involved in the coordination of type 1 copper, which has an important effect on the redox potential of the particular enzyme. This amino acid can either be a methionine (class 1), a leucine (class 2), or a phenylalanine residue (class 3) with redox potential (Hatamoto *et al.,* 1999).

The deduced amino acid sequences were compared with other fungal laccase sequences available in GenBank (Fig. 2). They displayed 66%~68% homology to the laccase from *Trametes versicolor*, and 53%~50%, 53%~50%, and 50%~47% homology to LAC1, LAC2, and LAC3 of *Letinula ed-*

odes, respectively. The regions of highest conservation are found in the copper binding domains (Necochea *et al.,* 2005). Finally, they are produced as isozymes encoded by a gene family. Generally, the true laccase isoforms are transcribed from separate genes, or are the result of post-translational modifications of extracellular polypeptides by proteolysis, or in glycosylation.

To elucidate the genomic organization of the laccase genes, the 3'-UTR segments (197 bp and 123 bp) of the two cDNAs were amplified by PCR, and used as specific probes on the Southern blots of the genomic DNA. The partial coding region of *pblac1* (557 bp), sharing 73% identity with *pblac2*, was obtained by PCR, and also used as another hybridization probe of *pblac1* (Fig. 3). The Southern analysis results of the *Xho*I-, *Eco*RV-, *Bam*HI-, and *Pst*I-digested genomic DNAs are shown in Fig. 3B. Only one strong band was observed from each restriction. We expected that the partial coding region probe would have shown at least two bands corresponding to *pblac1* and *pblac2* at each restriction, because the partial coding region showed a relatively high identity (73%) between *pblac1* and *pblac2*. The two probes, the partial coding region and 3'-UTR of *pblac1*, showed the same results, but a different band pattern from the 3'-UTR of *pblac2*. This indicated that both probes of *pblac1* were the specific probes of *pblac1,* and suggested that *pblac1* and *pblac2* were not closely related genes in the genomic DNA. We deem that some specific mechanisms of transcriptional activation are likely to be involved. An investigation on the mechanism of induction of mammalian cytochrome P-450c, in response to aromatic hydrocarbons, revealed xenobotic responsive elements (XREs) in the cytochrome P-450 promoter region (Fujisawa *et al.,* 1987). The XRE receptor or binding protein was a member of a large family of regulatory proteins that activate gene transcription in response to the presence of nonpolar carbon compounds (Fujisawa *et al.,* 1988). Also, the *lcc* promoter in the basidiomycete PM1 (Coll *et al.,* 1993) contained a sequence identical to the XRE consensus, 180 bp upstream of the TATA box. The presence of these putative XREs suggests that the transcription of these *lcc* genes was indeed activated by aromatic compounds such as DBP, which was utilized in this study.

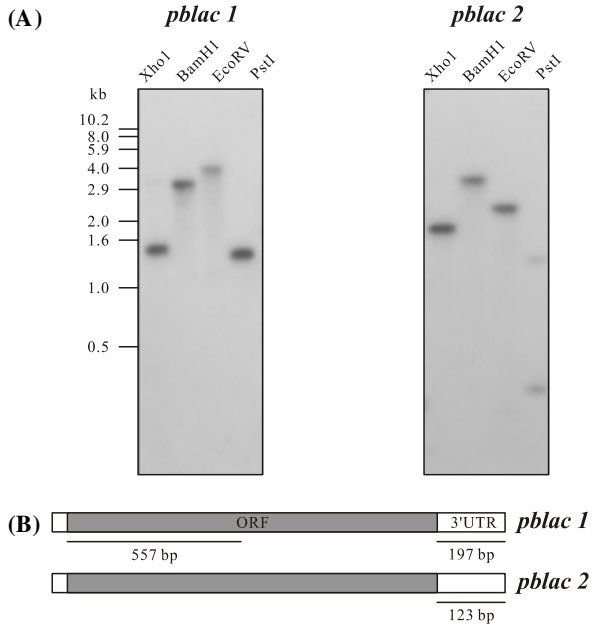

Fig. 3. Genomic Southern analysis of the laccase genes from *P. brumalis*. Equal amounts (25 μg) of genomic DNA were digested with the indicated restriction enzymes, fractionated by electrophoresis, transferred onto a nylon membrane, and probed with ^{32}P-labeled gene fragments: the 557 bp partial coding region fragment of *pblac1* (left of A) and the 123 bp 3'-UTR fragment of *pblac2* (right of A). Maps of the laccase genes are shown in (B) and the fragments used as probes are indicated with bars.

Competitive PCR analysis of gene expression

The two cDNAs had distinct sequences in their 3'-UTR regions. Thus, the gene-specific primers used for RT-PCR analysis were designed from the 3'-UTRs. The products of each RT-PCR were sequenced and identified as each lac-

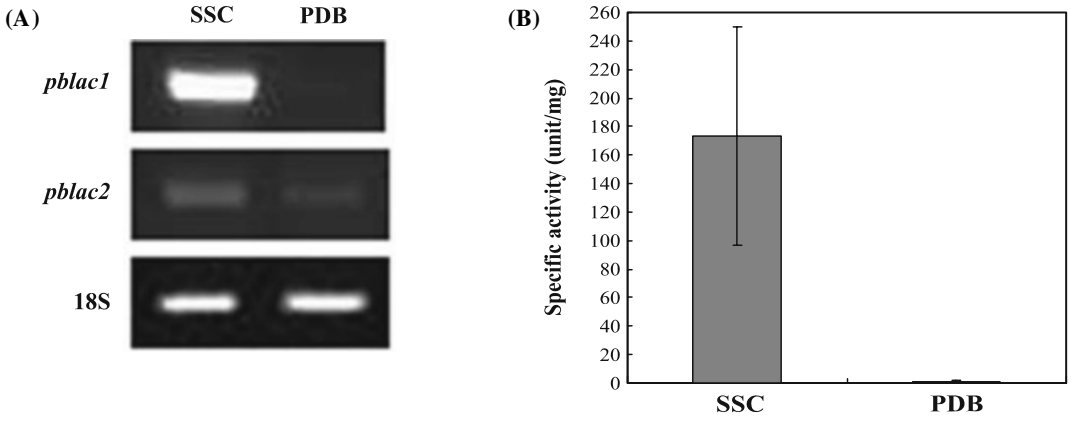

Fig. 4. RT-PCR analysis of the expression and specific activity of the laccase genes in *P. brumalis* mycelia cultured in different media. The RNA was extracted from 10 day-old liquid cultured *P. brumalis* mycelia in SSC medium and PDB medium. After reverse transcription was performed, as described by the kit manufacturer, PCR was performed using gene specific primers. The expression of the 18S rRNA genes by RT-PCR using commercial primers was performed to validate the concentration of cDNA. The reaction products (20 μl) were analyzed by gel electrophoresis.

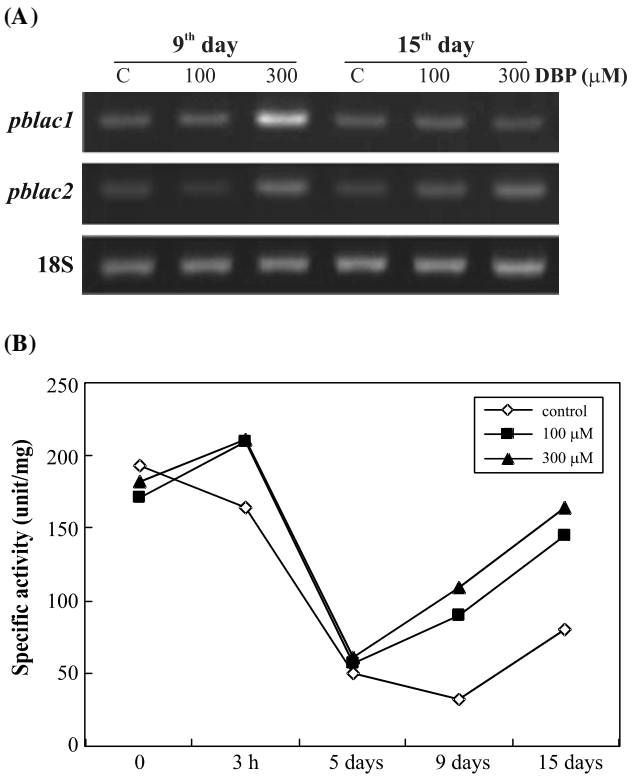

Fig. 5. Gene expression and specific activity of laccase in SSC liquid cultured *P. brumalis* mycelia in response to DBP. DBP (final conc. 100 μM and 300 μM) was applied to the liquid cultures at 5 days after pre-culture. The RNA was extracted on the 9th and 15th day after DBP treatment. C indicates no treatment. The expression of the 18S rRNA genes by RT-PCR using commercial primers was performed to validate the concentration of cDNA. The reaction products (20 μl) were analyzed by gel electrophoresis.

case, proving that the primers used were gene-specific. To investigate the effects of pertinent media on the production of the laccases, the expressions of the laccase genes were examined by RT-PCR analysis, using *P. brumalis* mycelia cultured in PDB and a SSC liquid medium (Fig. 4.). The results showed that the laccase genes were predominantly expressed in the SSC liquid medium, and the expression level of *pblac1* was higher than that of *pblac2* in this medium. The results for gene expression were identical to the tendencies of laccase specific activity. Thus, the laccase isozyme activity was controlled by the culture conditions at the transcriptional level. Laccase production can be influenced by the nitrogen concentration in the culture medium (Gianfreda *et al.,* 1999), as well as by the carbon source employed (Galhaup *et al.,* 2002). In this study, we demonstrated that nutrient nitrogen was provided to the fungus at limited concentrations (0.1% as compared to normal medium: 0.3~0.5%) in the SSC medium, and induced laccase production in *P. brumalis* at the level of gene transcription. Hence, it seems that nitrogen is an important factor in regulating laccase expression in white rot fungi.

It was established that the addition of DBP to *P. brumalis* cultures has a stimulatory effect on laccase production. The expression profiles of the laccase genes during DBP degradation in SSC media culture were examined with *pblac1* and *pblac2* gene specific primers (Fig. 5). Under the control condition, laccase expression proceeded at a gradual pace, achieving a maximum level when pre-grown for 5 days, and then declined thereafter (data not shown). Therefore, the 5 day-old pre-cultured mycelia were treated with DBP, and the induction of gene expression was investigated. As compared to the control, the expression levels of laccase increased at the 9th and/or 15th day in the cultures treated with DBP. In particular, *pblac1* treated with 300 μM DBP was highly expressed at the 9th day, but it did not increased at the 15th day. In the case of *pblac2* treated with 300 μM DBP, expression at both the 9th and 15th day was increased. The specific activities of the laccases treated with DBP were higher than those of the control culture on a whole. These results suggest that laccase expression was stimulated by DBP treatment.

The enzymes involved in the biodegradation of lignin and many aromatic xenobiotics, vary in white rot fungi. In a dye decolorization experiment with white rot fungi, laccase was the main enzyme in *Phlebia tremellosa* (Kirby *et al.,* 2000; Robinson *et al.,* 2001) and *Pleurotus sajorcaju* (Chagas and Durrant, 2001). *Trametes* species, well-characterized by laccase, have at least two isoforms, and the analysis of individual recombinant laccase isoenzymes has been achieved elsewhere (Cassland and Jönsson, 1999; Gelo-Pujic *et al.,* 1999; Larson *et al.,* 2001; Necochea *et al.,* 2005). Lee *et al.* (2005) researched the biodegradation of phthalic acid, a major metabolite in the biodegradation of phthalate esters, by *P. brumalis*. The concentration of phthalic acid in the culture medium was reduced after 4 days of incubation. After 24 days of incubation, no phthalic acid was detected. It was postulated from GC/MS analysis that the phthalic acid was consumed in a metabolic pathway related to glucose. In the biodegradation of di-butylphthalate (DBP) by *P. brumalis*, the biodegradation efficiency of DBP reached approximately 90% within 18 days of incubation (Lee *et al.,* 2005). From the results of preliminary experiments on *P. brumalis* in our lab, both MnP and laccase showed better activity than the control for the biodegradation of DBP (data not shown). This indicates that *P. brumalis* has various lignin degrading enzymes. However, it is difficult to analyze the individual contribution of the lignin degrading enzymes to a certain specificity or activity during degradation, which can be proposed by the molecular-biological properties that are the cloned and characterized genes of *P. brumails* involved in the biodegradation pathways of DBP. Furthermore, such information can be critical in the development of an advanced DBP degradation system, via modification and recombination using the cloned genes.

Overexpression of the *pblac1* gene using a homologous expression system in *P. brumalis*

We constructed transformants using a homologous system for the overexpression of a cloned laccase gene in *P. brumalis,* by the REMI method as described by Leem *et al*. (1999) with slight modifications. The transformants were selected from minimal medium plus hygromycin B, and transferred to new medium to examine their hygromycin resistance. To assess the production of laccase by the transformants, we

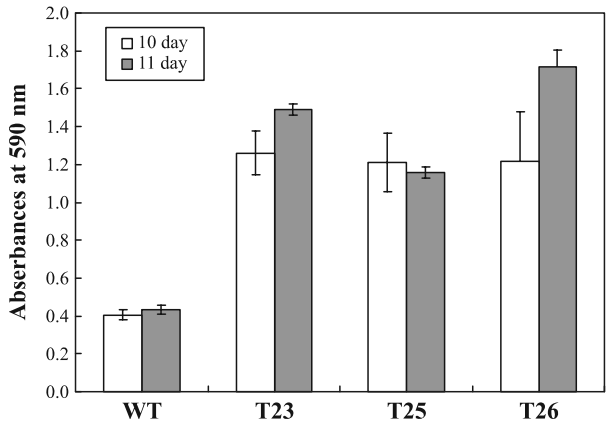

Fig. 6. Laccase activity based on *o*-tolidine oxidation by the wild type and transformants during culture in SSC medium. The formation of blue product(s) from the oxidation of tolidine, as measured by absorbencies at 590 nm, was directly proportional to the amount of extracellular laccase in the culture medium. Values are Mean±SE of three measurements with three replicates. Data outside the scale are annotated with the actual value of the measured absorbencies.

measured the absorbance of *o*-tolidine oxidation by laccase using a spectrophotometer at 590 nm. Increases in the absorbencies by the oxidation of *o*-tolidine showed laccase activity indirectly. As compared with the laccase activity of the wild-type strain, those of the transformants were 3∼4 times higher (Fig. 6). The expression of the recombinant laccase of the *pblac1* cDNA was derived by the constituted promoter of a gene for glyceraldehyde-3-phosphate dehydrogenase in the transformants, and revealed a higher expression than that of the wild type. Although we have not yet confirmed the properties of the recombinant protein, such as its molecular weight and specific laccase activity, the yield of laccase from the transformants with a cloned gene was elevated, and is expected to contribute to the utilization of bioremediation.

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