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목질계 바이오매스의 생물학적 전처리를 위한 겨울우산버섯 유래 리그닌분해효소의 클로닝 및 해석

Cloning and characterization of lignin degrading enzymes in *Polyporus brumalis* for biological pretreatment of lignocellulosic biomass

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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Cloning and characterization of lignin degrading enzymes in *Polyporus brumalis* for biological pretreatment of lignocellulosic biomass

Advisor: Professor Jin-Ho Seo

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ABSTRACT

Cloning and characterization of lignin degrading enzymes in *Polyporus brumalis* for biological pretreatment of lignocellulosic biomass

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The genes encoding lignin degradation-related enzymes including laccase and manganese peroxidase (MnP) were isolated and overexpressed in a white-rot fungus, *Polyporus brumalis*, followed by functional analysis of the transformants.

Two laccase (*pblac1* and *pblac2*) and six MnP (*pbmnp1-6*) cDNAs were cloned from *P. brumalis* (KFRI 20912) which was isolated by the Korea Forest Research Institute. The deduced amino acid sequences of the laccase and MnP genes shared 70% and 62-96% identity, respectively. An RT-PCR analysis indicated that the RNAs of these

genes were predominantly expressed in shallow stationary culture (SSC) in a liquid medium and increased after treatment of dibutyl phthalate (DBP) and wood chips. Especially, the transcription levels of *pblac1* and *pbmnp4* were higher than those of other genes and were proportional to the corresponding enzyme specific activity, suggesting that the transciption level of the two genes plays an important role in enzyme activity.

Both *pblac1* and *pbmnp4* genes under the control of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter were overexpressed by genetic transformation in *P. brumalis*. The enzyme activities of both laccase and MnP in the transformants were significantly higher than those of the wild type. The transformants exhibited more effective decolorization of the dye Remazol Brilliant Blue R than the wild type.

When incubating with wood chips from red pine (softwood) and tulip tree (hardwood) for 15 and 45 days, the transformant with enhanced laccase activity showed higher lignin-degrading activity as well as higher wood-chip weight loss than the wild type. When the wood chips treated with the transformant were enzymatically saccharified, the highest sugar yields were found to be 32.5% for red

pine wood and 29.5% for tulip tree wood based on the dried wood weights that were about 1.6-fold higher than those for the wild type. These results suggested that overexpression of the laccase gene from *P. brumalis* significantly contributs to the pretreatment of lignocellulose for increasing sugar yields.

Thus, the identification of the genes for laccase and MnP cDNAs is the first step to characterize the molecular events related to the lignin degradation ability of white-rot fungi, which can contribute to the efficient production of lignin degradation enzymes and lead to the utilization of these fungi for the biological pretreatment of lignocellulosic biomass.

Keywords : *Polyporus brumalis*, laccase, manganese peroxidase (MnP), lignocellulosic biomass, transformant, pretreatment

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CHAPTER I

General introduction

1.1. Background

Development of renewable energy is required because of recent global warming and high prices of fossil fuels. Bioethanol produced from biomass to replace fossil fuels has emerged as energy in the transportation sector. Global bioethanol production in 2010 was 85.8 billion liters and it is expected to reach 100 billion liters in 2015 (Taherzadeh and Karimi, 2007; Mojović et al., 2012). Bioethanol is currently produced by fermentation using corn glucose in the United States, or sucrose from sugar cane in Brazil. Since sugar cane and corn are food crops (Smith, 2008), bioethanol production may lead the price of crops to higher levels in the future, which are caused by limitation in feedstock supply (Gray et al., 2006) and competition with food and feed markets (Ajanovic, 2011).

Lignocellulosic biomass is a promising feedstock for the production of fuel ethanol, because it is renewable and abundantly available. Lignocellulosic materials can be obtained at low cost from a variety of resources, e.g. forest and agricultural residues. Bioethanol obtained from lignocellulosic materials is favorable from a greenhouse gas perspective, with around an 85% net reduction in greenhouse gas compared to gasoline (Farrell et al., 2006). The first step in the

conversion of lignocellulosic biomass for ethanol production is pretreatment, which is performed by breakdown of the lignin barrier to liberate cellulose and hemicellulose and allows for efficient enzymatic hydrolysis (Mosier et al., 2005).

Pretreatment of lignocellulosic biomass can be performed by physical and mechanical, chemical, and biological methods (Moiser et al., 2005; Taherazadeh and Karimi, 2008; Hu et al., 2008; Hendriks and Zeeman, 2009; Alvira et al., 2010). Physical and mechanical pretreatments are based on milling, irradiation and hydrothermal treatments. Chemical pretreatments include alkali, acid and organosolve treatments. Physical, mechanical and chemical methods effectively reduce biomass recalcitrance in short time and are thus attractive for industrial applications. However, these methods require high-energy and/or corrosion-resistant high-pressure reactors, and extensive washing, which increase the cost of pretreatment. Furthermore, chemical methods may produce toxic substances, interfering with the subsequent microbial fermentation, in addition to producing waste water that needs treatment prior to its release to the environment (Keller et al., 2003; Shi et al., 2008).

Biological pretreatment has attracted interests because of its potential

advantages over physical and chemical methods such as a greater substrate and reaction specificity, lower energy requirements, lower pollution generation, and higher yields of desire products (Isroi et al., 2011). Compared to physical and chemical pretreatments, biological pretreatements have been less investigated. The reason for this fact could be slow rates of the pretreatments and the potential carbohydrate loss because of cellulose and hemicelluloses degradation. However, biomass pretreatment for production of bioethanol as an alternative energy to replace fossil fuels demands an environmentally friendly process.

Biological pretreatment employs microorganisms such as white-rot fungi and their enzymatic machineries to break down lignin and alter lignocelluloses structures. White-rot fungi, which decay the wood in forest, can mineralize lignin to CO_2 and water in pure culture (Lundquist et al., 1977; Hatakka, 1983) using several ligninolytic enzymes such as laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP). Thus, there has been an increasing interest in the investigation for enhancing biological pretreatment by white-rot fungi. Identification of ligninolytic enzymes can contribute to enhancing the ability of lignin degradation and improving the efficient of pretreatment.

1.2. Literature review

1.2.1. White-rot fungi

A wood-decay fungus is digests moist wood, causing it to rot. They can be classified into three main groups according to the type of decay that they cause, as brown-rot, soft-rot, and white-rot fungi (Schwarze et al., 2000). Brown-rot fungi break down hemicellulose and cellulose. As a result of this type of decay, the wood shows a brown discoloration, and cracks into roughly cubical pieces. Soft-rot fungi secrete cellulase from their hyphae, an enzyme that breaks down cellulose in the wood. This leads to the formation of microscopic cavities inside the wood, and sometimes to a discoloration and cracking pattern similar to brown rot. White-rot fungi break down the lignin in wood, leaving the lighter-colored cellulose behind; hence, the names white rot. Some of them break down both lignin and cellulose. Because white-rot fungi are able to produce extracellular ligninolytic enzymes such as lignin peroxidase (LiP, E.C. 1.11.1.14), manganese peroxidase (MnP, E.C. 1.11.1.13) and laccase (E.C. 1.10.3.2), they have been investigated for mycoremediation applications (Cohen et al., 2002). Biological pretreatment of lignocellulosic biomass with white-rot fungi has previously been investigated for paper-related applications. Recently,

this environment-friendly approach has received renewed attention as a pretreatment method for enhancing enzymatic saccharification in biofuel production (Isroi et al., 2011).

1.2.2. General properties of ligninolytic enzymes

LiP and MnP were discovered in *Phanerochaete chrysosporium* almost 30 years ago (Glenn et al., 1983; Tien and Kirk, 1983; Kuwahara et al., 1984). These enzymes have been demonstrated to be major components of the lignin degradation system of this organism. They are heme-dependent peroxidases that are believed to initiate free-radical reactions in which the radical generated may attack the lignin network. The catalytic cycles of both LiP and MnP resemble those of other heme peroxidases such as horseradish peroxidase (HRP) (Gold et al., 1989). The catalytic cycle of LiP is initiated by binding of H_2O_2. The primary reaction product is the two-electron oxidized state compound I (LiPI). This is reduced back to the native enzyme via two single-electron steps with compound II (LiPII), as an intermediate. In the process, the aromatic reducing substrate is oxidized to an aryl cation radical (Gold and Alic, 1993).

$LiP + H_2O_2 \rightarrow LiPI + H_2O$

$LiPI + Ar \rightarrow LiPII + Ar^{+\bullet}$

$LiPII + Ar \rightarrow LiP + Ar^{+\bullet} + H_2O$

LiP catalyzes the H_2O_2-dependent oxidation of nonphenolic lignin model compounds, aromatic pollutants and synthetic lignin (Gold et al., 1982; Miki et al., 1988; Hammel and Moen, 1991; Valli et al., 1992).

The catalytic cycle of MnP is also initiated by binding of H_2O_2, but the primary reducing substrate is Mn(II) (Glenn and Gold, 1985; Gold et al., 1989). This reaction efficiently reduces both compound I (MnPI) and compound II (MnPII), generating Mn(III), which subsequently oxidizes the organic substrate (Gold and Alic, 1993).

$MnP + H_2O_2 \rightarrow MnPI + H_2O$

$MnPI + Mn(II) \rightarrow MnPII + Mn(III)$

$MnPII + Mn(II) \rightarrow MnP + Mn(III) + H_2O$

MnP catalyzes the H_2O_2-dependent oxidation of lignin, lignin derivatives and a variety of phenolic lignin model compounds (Gold et al., 1989; Wariishi et al., 1988; Lakner et al., 1991; Tuor et al., 1992).

Orgnic acids, such as oxalate and malonate, stimulate the MnP reaction by stabilizing the Mn(III) to form stable complexes with a redox potentials so that Mn ion can participate in the reaction as diffusible redox couple and oxidize the insoluble therminal substrate including lignin (Glenn and Gold 1985; Glenn et al., 1986; Gold et al., 1989; Warlishi et al., 1989; Warlishi et al., 1992).

Laccase was first described in the varnish tree *Rhus vernicifera* by Yoshida in 1883; however, most laccases have been found and studied in lignin-degrading basidiomycetes, white-rot fungi (Yoshida et al., 1883; Reinhammar et al., 1984; Thurston et al., 1994). Unlike the peroxidases, it does not contain heme as a cofactor but copper and it also does not need H_2O_2 as a substrate but rather molecular oxygen (Baldrian, 2006). Laccase can catalyze the 4-electron reduction of O_2 to H_2O. Laccase attacks the phenolic substrates with the use of O_2 and oxidizes non-phenolic lignin, which is resistant to LiP and MnP attack through redox mediators (Majcherczyk et al., 1998; Srebonik et al., 1998; Li et al., 1999). Consequently, these ligninolytic enzymes have a wide range of nonspecific degradation ability. Laccase catalysis is believed to take place in three steps: 1. Type I Cu reduction by substrate. 2. Electron transfer from type I Cu to the type II Cu and type

III Cu trinuclear cluster. 3. Reduction of oxygen to water at the trinuclear cluster (Gianfreda et al., 1999). The substrate range of laccase can be extended to non-phenolic subunits by the inclusion of mediators (Gochev and Krastanov, 2007). Mediators are a group of low molecular-weight organic compounds that can be oxidized by laccase first and from highly active cation radicals to reach with carious chemicals including non-phenolic compounds that laccase alone can not oxidise (Figure 1-1).

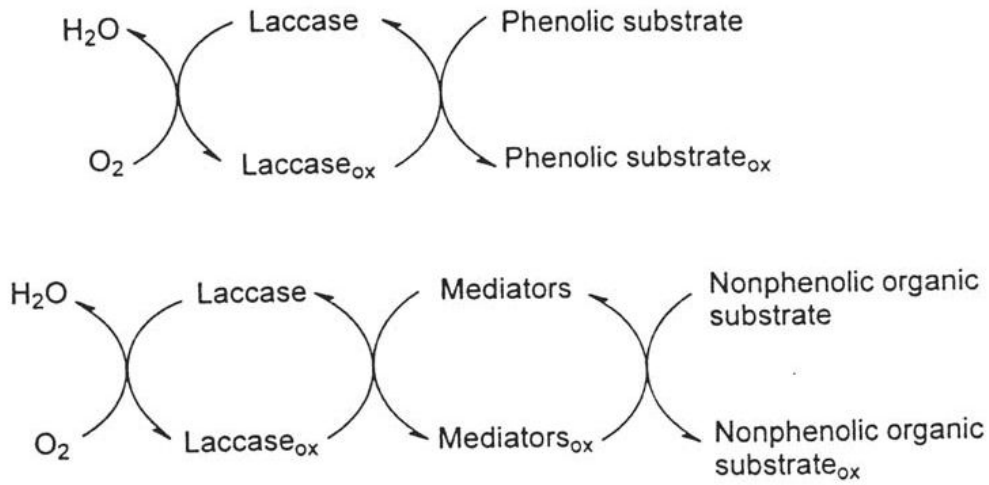

Figure 1-1. Catalytic mechanism of laccase with and without mediators (Gochev and Krastanov, 2007).

1.2.3. Biological pretreatment of lignocellulosic biomass using white-rot fungi

Lignin is a complex aromatic amorphous polymer derived from lignocellulosic biomass such as wood, and an integral part of the secondary cell walls of plants. Because lignin acts as obstacles in the hydrolysis of cellulose and hemicellulose, the pretreatment process, destruction of lignin structure, allows for efficient hydrolysis (Mosier et al., 2005). Thus, pretreatment is the first step in producing bioethanol from lignocellulosic biomass (Balat et al., 2010). Chemical and physical pretreatments are effective to reduce lignin in short time and are thus attractive for industrial applications. However, these methods have economic and environmental problems such as high-energy consumption and emissions of toxic substances (Keller et al., 2003; Shi et al., 2008). Thus, biological pretreatment using white-rot fungi has received renewed attention as the environmental-friendly approach recently. Several white-rot fungi have been investigated for pretreatment to improve hydrolysis of various lignocellulosic biomass.

Taniguchi et al. (2005) reported that the amount of total soluble sugar obtained from the rice straw pretreated with *Pleurotus ostreatus* for 60 days was 33%, which was 2.4 or 3.4 times higher than that from

untreated rice straw. Yu et al. (2009b) also used *P. ostreatus* for the pretreatment of rice hull to increase hydrolysis of biomass feed stocks with a maximum sugar yield of 36.7% after 60 days treatment. Furthermore, they combined biological pretreatment with chemical pretreatment to show that the combined pretreatment with H_2O_2 for 45 h and *P. ostreatus* for 18 days was more effective than a sole pretreatment of *P. ostreatus* for 60 days. Yu et al. (2009a) pretreated two types of woods, hardwood and softwood. Pretreatment with *Echinodontium taxodii* 2538 for 90 days has been found to enhance the enzymatic hydrolysis rate of hardwood (Chinese willow) by a 4.7 fold and softwood (China fir) by a 6.3 fold (Yu et al., 2009a). Lee et al. (2007a) compared the effects of the biological pretreatment by three white-rot fungi *Ceriporia lacerate, Stereum hirsutum,* and *P. brumalis* with wood chips of *Pinus densiflora*. The highest total sugar yield (21.01%) was obtained when saccharification was performed with pretreated wood by *S. hirsutum.* Total sugar yield of pretreated wood by *C. lacerate* and *P. brumalis* were 15.03% and 14.91% under the same condition, respectively. Of the three fungi tested, *S. hirsutum* was considered as a suitable fungus for biological pretreatment (Lee et al., 2007a). Wan et al. (2010) investigated the effect of particle size and

moisture content of biomass, pretreatment time, and temperature on the pretreatment of corn stover with *Ceriporiopsis subvermispora*. The highest glucose yield was 66.61% when 5 mm corn stover was pretreated at 28℃ with 75% moisture content for 35 days (Wan et al., 2010).

1.2.4. The use of P. brumalis

P. brumalis is classified as a group of polyporaceae basidiomycetes, which causes white rots on dead hard wood and stumps. This fungus distributs widely over the world including Korea. Lee et al. (2005) studied the degradation mechanism of dibutyl phthalate (DBP) and di (2-ehylhexyl) phthalate, abundantly used as additives in the plastic industry, by *P. brumlais*. They used this fungus because *P. brumalis* has been selected from the screening of domestic wood-rot fungi for biodegradation of hazardous recalcitrant chemicals and showed the good ability in lignin degradation and the strong resistance to the various recalcitrant toxic pollutants (Lee et al., 2005; Lee et al., 2007b). *P. brumalis* completely degraded 1.2 mM DBP after the 18-day incubation period (Lee et al., 2007b). Nakade et al. (2010) screened several species of the polyporaceae in order to find strains capable of

producing a large amount of laccase with high redox potential comparable to that of *Trametes versicolor* which is well known to possess as high laccase activity (Xiao et al., 2003; Han et al., 2005). From this screening, they reported a strain of *P. brumalis* able to produce a high amount of laccase reaching 7.72 U/mL. The purified laccase showed 0.8 V ± 1.001 E° value versus a normal hydrogen electrode, which is a very high redox potential compared to those of other basidiomycetous laccase (Nakade et al., 2010).

1.3. Objectives of the dissertation

The purpose of this study is to improve the biological pretreatment of white-rot fungi via genetic transformation for increasing the bioethanol production yield from lignocellulosic biomass. Several researchers pretreated lignocellulosic biomass with various fungi to improve the pretreatment performance. However, these studies have focused on the improvement of pretreatment processes and the selection of effective fungi with high ligninolitic activity. This dissertation was focused on the development of new strains with enhanced ligninolytic activity through overexpression of lignin-degrading genes and the potential of the genetic transformants for biological pretreatment of lignocellulosic biomass. The specific objectives of this research were as follows:

1) To isolate useful genes involved in lignin degradation from a white-rot fungus, *P. brumalis* and to construct strains with enhanced ligninolytic activity using a homologous genetic transformation system.
2) To analysis the efficiency of lignin-degradation and saccharification when lignocellulosic biomass were pretreated with the genetic transformants.

CHAPTER II

Molecular characteristics of two laccase cDNAs from a basidiomycete fungus, *Polyporus brumalis*

2.1. Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to the copper-containing oxidases. It was first found in the varnish tree *R. 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 an 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 (Martirani et al., 1996; Palmieri et al., 1993; Reid and Paice, 1994; Han et al., 2004; Van Aken et al., 1997).

So far, laccase genes have been isolated by molecular cloning from some white-rot fungi such as *T. versicolor* (Ong et al., 1997; Cassland

and Jonsson 1999; Cheong et al., 2006), *C. 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 characterized.

P. 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 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, the nucleotide sequences of laccase genes were analyzed from *P. brumalis*. Then, the

expression patterns were investigated in the fungus, and transformants were constructed using a homologous genetic transformation system for the overexpression of the cloned laccase genes in *P. brumalis*.

2.2. Materials and Methods

2.2.1. Fungal cultures and DBP treatment

The *P. brumalis* 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, $MgSO_4.7H_2O$ 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, $FeSO_4.7H_2O$ 0.1 g, $CoCl_2$ 0.1 g, $ZnSO_4.7H_2O$ 0.1 g, $CuSO_4$ 0.1 g, $AlK(SO_4)_2.12H_2O$ 10 mg, nitriloacetic acid 1.5 g in distilled water 1,000 mL) in distilled water, 1,000 mL] at 25℃ (Tien and Kirk, 1984). For the DBP treatments, *P. brumalis* was pre-grown in SSC liquid medium for 5 days at 25℃, and treated with 100 and 300 μM DBP, and then incubated for 9 days.

2.2.2. Specific activity assay of laccase with ABTS

Specific activity of laccase 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 contained 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 an enzyme oxidizing 1 μmol of ABTS min^{-1} (ε = 36,000 M^{-1} cm^{-1}). The kinetic studies were performed by measuring the initial rate of the enzyme-catalyzed reactions measured at 414 nm for ABTS. The protein assay was determined by the Bradford method (Bradford, 1976) with 1 mg mL^{-1} of bovine serum albumin (BSA; Sigma, MO, USA)

2.2.3. Cloning of laccase genes by RT-PCR

The total RNA was extracted with the 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 200units of MMLV reverse transcriptase (Promega, USA), as recommended by the manufacturer. Approximately 1 kb fragments were amplified with pairs of degenerated oligonucleotide primers (5'-CAYTGGCAYGGNTTYTTYCA-3', 5'-GHWMBTHYTGGTRBCACWSYC-3') designed from the conserved regions of the previously reported laccase sequences at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/protein); Tv-LAC (*T.*

versicolor): BAD98308, Le-LAC1 (*L. edodes*): AAT99287, Le-LAC2 (*L. edodes*): BAB83132, Le-LAC3 (*L. edodes*): AAT9929 (Nagai et al., 2002; Fujihiro et al., 2009). PCR was performed as follows: initial denaturing at 94°C for 120 s, 30 cycles of denaturing at 94°C for 60 s, annealing at 55°C for 30 s, elongation at 72°C for 90 s, and ending with 10 min final extension at 72°C. The amplified fragments were subcloned into a pCR2.1TOPO (Invitrogen, USA) vector, and sequencing of the double-stranded plasmid DNA was performed.

2.2.4. Rapid amplification of cDNA ends (RACE)

RT reactions from 1 μg of purified total RNA were performed 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 *pblac1*: 5'-GGACCGAGCCTAGCAGCAACG-3', 5'-CGTCATTACACTGGCAGATTGG-3'; and *pblac2*: 5'-CTGACCGGCGTAGATCTGGATG-3', 5'-GACCATCGAGCTCTCCTTCCCG-3') and inner (nested) gene coding sequence specific primers (NGSPs of *pblac1*: 5'-CCAATCTGCCAGTGTAATGACG-3', 5'-

CGTTGCTGCTAGGCTCGGTCC-3'; and *pblac2*: 5'-CATCGACAACCCCACCAAAAAAG-3', 5'-CACCCCTTCCATCTGCACGG-3') that were designed for *pblac1* and *pblac2*. The PCR conditions were as follows: 5 cycles of denaturing at 94°C for 30 s, annealing and elongation at 72°C for 180 s, 5 cycles of denaturing at 94°C for 60 s, annealing at 70°C for 30 s, elongation at 72°C for 180 s, 25 cycles of denaturing at 94°C for 60 s, annealing at 68°C for 30 s, and finally, elongation at 72°C for 180 s. 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 full length cDNAs have been deposited in GenBank under the accession numbers EF362634 (*pblac1*) and EF362635 (*pblac2*).

2.2.5. Determination of gene expression

An RT-PCR kit (Promega, USA) was used to identify the mRNA coding for laccase, and an 18S rRNA served as an 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', 5'-AGTCGCAATTTGCTTCG-3') generated a 199 bp product, and the *pblac2* primer set (5'-GCGGACTGCTGTATCTC-3', 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.

2.2.6. 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 weight and signal peptides of the deduced proteins the ExPasy program (http://www.expasy.org/tools/) was used. A sequence alignment was constructed with the ClustralX program (http://www.clustal.org).

2.2.7. Genomic 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, USA). 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).

2.2.8. Generation of P. brumalis transformants by REMI

A pHYlac1 vector was constructed 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 a selectable maker, which contained the *pblac1* cDNA under the control of the *gpd* promoter (Figure 2-1). The transformation of *P. brumalis* was performed by the REMI method as described by Leem et al. (1999) with slight modifications. After mycelia of *P. brumalis* were cultured at 25℃ for 1 day in CKMM medium (maltose 10 g, KNO_3 2 g, trace element solution 40 mL, and salt solution 25 mL in H_2O 1,000 mL. Trace element solution consisted of $CaCl_2$ 2.73 g, $MgCl_2 \cdot 6H_2O$ 10.25 g, $FeCl_3 \cdot 6H_2O$ 1.33 g, citric acid 1.33 g, $MnSO_4$ 1.1 g and $ZnSO_4$ 1.0 g in H_2O 1,000 mL. Salt solution consisted of ammonium tartrate 10 g, KH_2PO_4 20 g, Na_2HPO_4 45 g and

Na_2SO_4 5.6 g in H_2O 500 mL). The mycelia were collected, washed twice with 1% sucrose, and treated with 0.5% USUKizyme (Kyowa Chemical Products Co. Ltd., Osaka, Japan) in 1% sucrose at 30℃ for 3 h with shaking at 80 rpm. After incubation, the protoplasts in the suspension were separated from cell debris by filtration through a miracloth and then collected by centrifugation at 3000 rpm for 5 min at 4℃. They were suspended in STC buffer (0.01 M Tris-HCl, pH 7.5, 0.01 M $CaCl_2$, 1.2 M sorbitol). Transformation was performed using 40% polyethylene glycol (PEG 400; Sigma, USA). The plasmid (10 μL of 1 μg mL^{-1}) was added to 100 μL of protoplast suspension in an Eppendorf tube. After 30 min incubation on ice, 1 mL of 40% PEG 400 in 25 mM $CaCl_2$ and 25 mM Tris-HCl, pH 7.5 was added and the mixture was incubated at room temperature for 20 min.

The transformants were selected from minimal medium containing 50 μg mL^{-1} hygromycin B, and transferred to new medium containing 100 μg mL^{-1} hygromycin B to examine their hygromycin resistance.

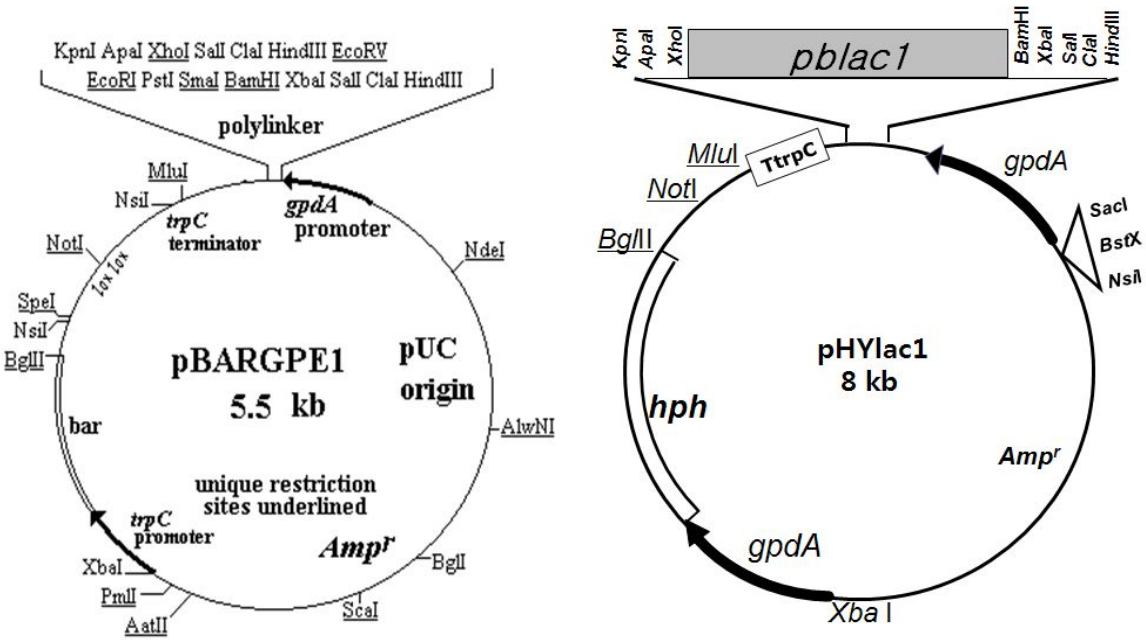

Figure 2-1. Restriction map of the transforming plasmid pBARGPE1 and pHYlac1. The sequence encoding laccase gene, *pblac1,* is under the control of the *gpd* promoter (*gpdA*). A hygromycine resistance gene was indicated as *hph*.

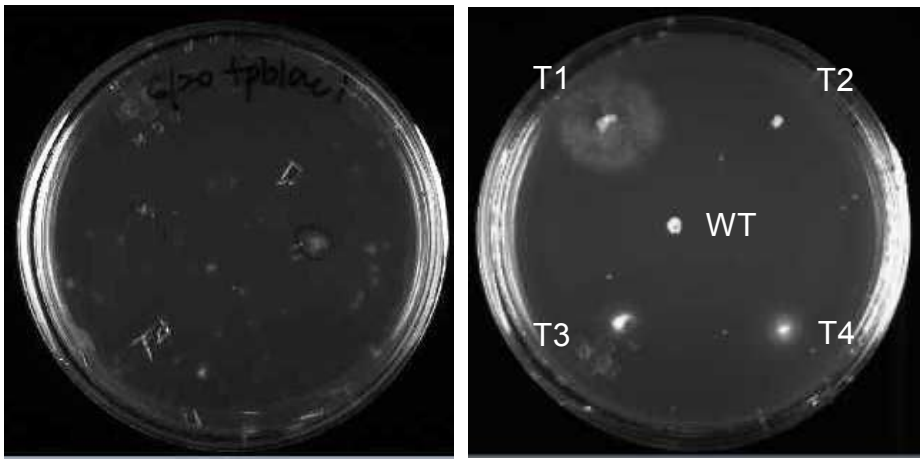

Figure 2-2. Selection of transformants generated with pHYlac1. The 1st selection medium contains 50 μg mL^{-1} hygromycin B (left) and the 2nd selection medium contains 100 μg mL^{-1} hygromycin B (right). WT, wild type strain; T1-T4, represent the transformants.

2.2.9. Confirmation of genomic integration in P. brumalis transformants

The genomic DNA from the transformants and the wild type strain was used as the PCR template and the *hph* gene was amplified using the following primers: 5′-TGGATATGTCCTGCGGGTAA-3′ as the forward primer and 5′-CGTCAGGACATTGTTGGAGC-3′ as the reverse primer.PCR was performed as follows: initial denaturing at 94°C for 120 s, 30 cycles of denaturing at 94°C for 60 s, annealing at

60°C for 30 s, elongation at 72°C for 60 s, and ending with a 10 min final extension at 72°C.

2.2.10. Comparison of laccase activities with o-tolidine

The laccase activity of the wild type and transformants grown in potato dextrose broth (PDB) were measured using a modified version of the Ross Method (Ross, 14982) with *o*-tolidine as a chromogenic enzyme substrate. Stock solutions of 4.7 mM *o*-tolidine were prepared in 95% ethanol with 67 mM glycine and 2 mM glacial acetic acid. Assays were carried out in a 96 well multi-micro plate in a total volume of 300 μL at room temperature. The reaction was initiated by adding 7.5 μL of liquid culture medium containing laccase and then absorption spectra were taken following 5 min incubation at 590 nm. One enzyme unit was defined as the amount of an enzyme which generated 0.1 O.D. under the above conditions. The protein concentration was determined by Bradford methods (Bradford, 1976). The transformant strains were maintained on potato dextrose agar (PDA) containing hygromycin.

2.2.11. Comparison of decolorization with Remazol Brilliant Blue R dye

The assay system (volume, 300 μL) used contained a filtrate of crude broth, obtained after 6 days incubation of each transformant and wild type strain in liquid culture in PDB media, 100 μg mL^{-1} Remazol Brilliant Blue R (RBBR). After 5 h incubation at room temperature, the absorption of residual RBBR at 590 nm was determined to examine a capacity for fungal decolorization.

2.3. Results and Discussion

2.3.1. 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 based on the conserved sequences. By using RT-PCR methods, 2 PCR products of 1,152 bp and 1,176 bp (referred to as *pblac1* and *pblac2*) were obtained (Figure 2-3). When the fragment sequences were analyzed 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* 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. 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*).

2.3.2. Sequence analysis of laccase genes

The nucleotide sequences of the cDNAs encoding laccase and their deduced amino acid sequences are shown in Figure 2-3. 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). Table 2-1 gives an overview of the results of amino acid sequence comparison between the products of laccase cDNAs. The similarity between *pblac1* and *pblac2* is 70%, and they show 47-68% homology with other laccases which isolated from *T. versercular* and *L. eddodies*. Both genes have putative N-glycosylated sites at the amino acid positions 74 and 354**,** 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).

Table 2-1. Pairwise comparison of the laccases encoded by cDNAs isolated from *P. brumalis* and previously reported

Protein[a]	*pblac1*	*pblac2*	Tv-LAC	Le-LAC1	Le-LAC2
pblac1	-	-		-	-
pblac2	70	-	-	-	-
Tv-LAC	66	68	-	-	-
Le-LAC1	53	50	53	-	-
Le-LAC2	53	50	53	98	-
Le-LAC3	50	47	50	64	64

[a]The values indicate the degree of amino acid sequence identity between the six laccases. Gene accession No. of Tv-LAC (*T. versicolor*): BAD98308, Le-LAC1 (*L. edodes*): AAT99287, Le-LAC2 (*L. edodes*): BAB83132, Le-LAC3 (*L. edodes*): AAT9929.

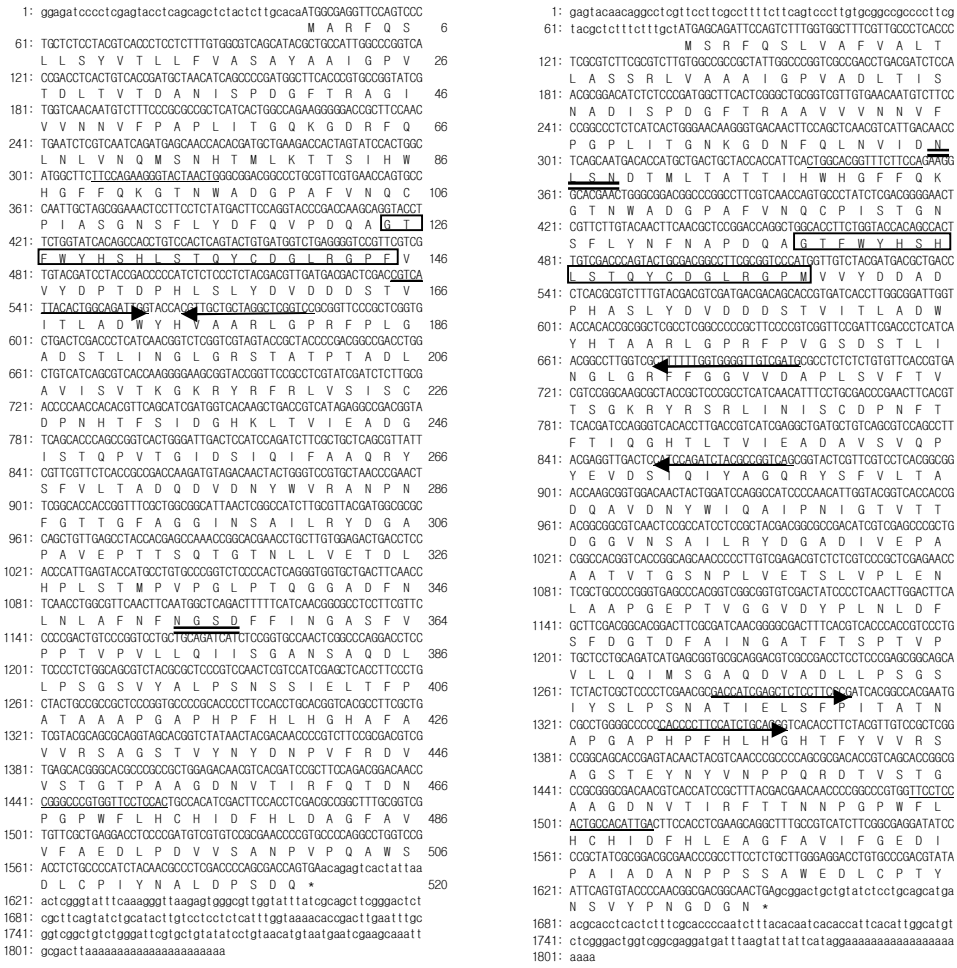

Figure 2-3. DNA and deduced amino acid sequences of the laccase genes, *pblac1* (left) and *pblac2* (right). The primer sequences for the confirmation of laccase by RT-PCR are indicated by the underlines. Sequences with arrows represent the primers used for RACE. The possible N-glycosylation sites are marked by double lines, and the multi-copper oxidase signature sequences are indicated in boxes.

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 amino acids 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 (Figure 2-4). They displayed 66-68% homology to the laccase from *T. versicolor*, and 50-53%, 50-53% and 47-50% homology to LAC1, LAC2 and LAC3 of *L. edodes,* 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.

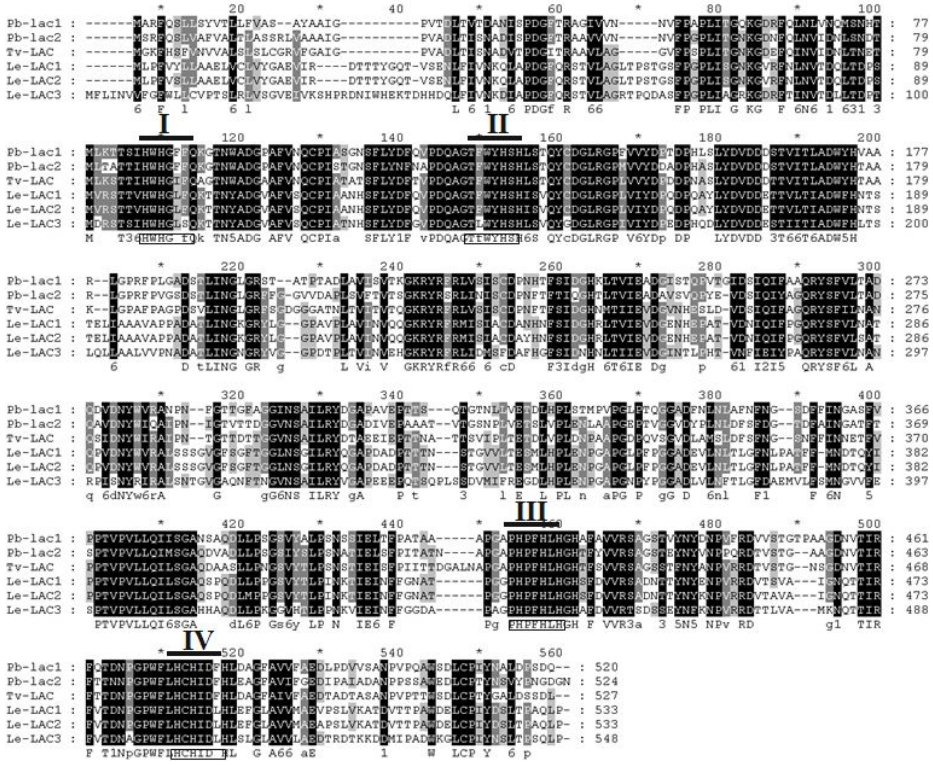

Figure 2-4. Alignment of the deduced *pblac1 and pblac2* amino acid sequences with those of other white-rot fungi laccases. Four potential copper-binding domains are indicated by horizontal lines (I, II, III and IV). The accession numbers of the sequences are as follows: Tv-LAC (*T. versicolor*), BAD98308; Le-LAC1 (*L. edodes*), AAT99287; Le-LAC2 (*L, edodes*), BAB83132; Le-LAC3 (*L. edodes*), AAT99291. The alignment was performed with ClustalX1.81 software.

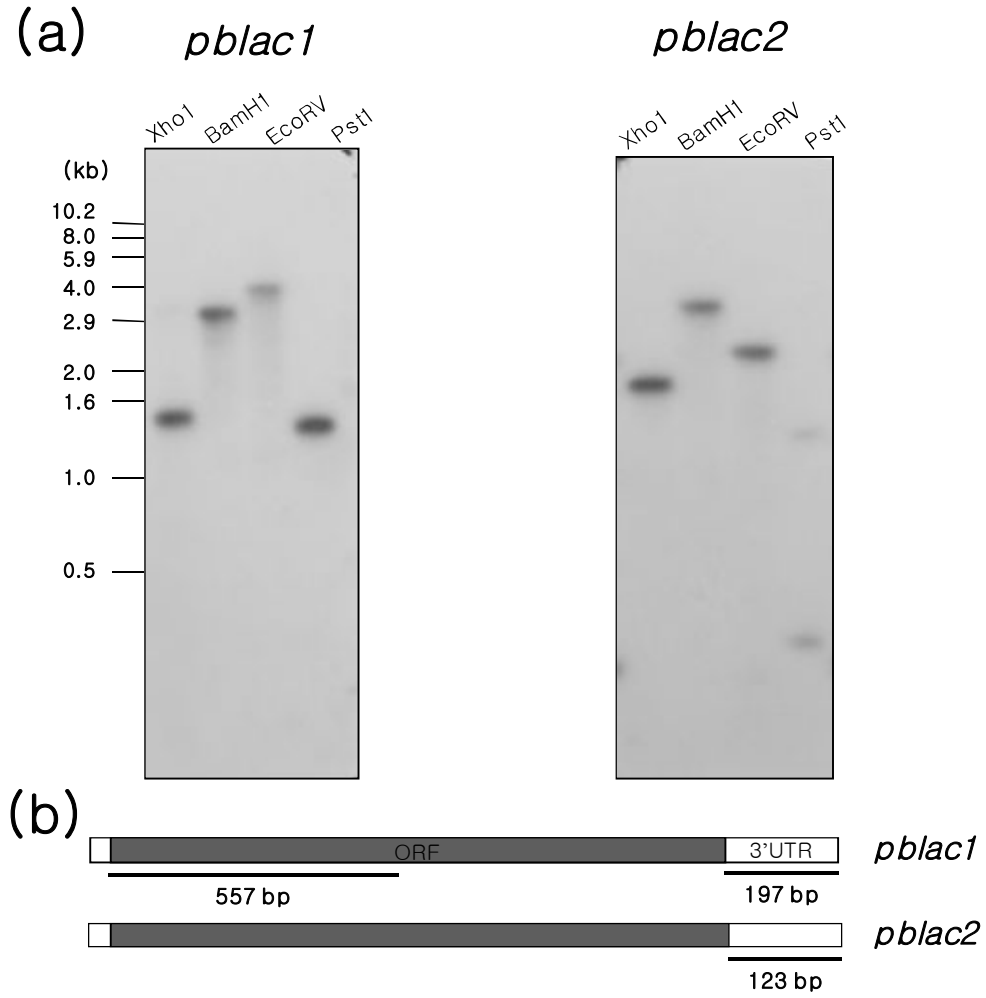

Figure 2-5. Genomic Southern analysis of the laccase genes from *P. brumalis*. Equal amounts (25 μg) of genomic DNA were digested with the indicated restriction enzymes, fractionated by electrophoresis, transferred onto a nylon membrane, and probed with ^{32}P-labeled gene fragments: the 557 bp partial 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.

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* (Figure 2-5). The Southern analysis results of the *Xho* I-, *Eco* RV-, *Bam* HI-, and *Pst* I-digested genomic DNAs are shown in Figure 2-5b. Only one strong band was observed from each restriction. It is 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.

There is supposed 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 laccase 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 the laccase genes was indeed activated by aromatic compounds such as DBP.

2.3.3. 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 laccase, proving that the primers used were gene-specific. To investigate the effects of pertinent media on the production of the laccases, the expression of the laccase genes was examined by RT-PCR analysis, using *P. brumalis* mycelia cultured in

PDB and a SSC liquid medium (Figure 2-6). 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). This study 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 demonstrated 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 (Figure 2-7). 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 five 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 9^{th} day in the cultures treated with DBP. In particular, *pblac1* treated with 300 μM DBP was highly expressed. The specific activities of the laccases treated with DBP were higher than that of the control culture. These results suggested 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 for laccase, have at least two isoforms, and the analysis of individual recombinant laccase isoenzymes has been achieved elsewhere (Necochea et al., 2005; Larson et al., 2001; Cassland et al., 1999; Gelo-Pujic et al., 1999). Lee et al. (2005) studied 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 95% within 12 days of incubation (Lee et al., 2005). From the results of preliminary experiments on *P. brumalis*, both MnP and laccase showed better activity than the control for the biodegradation of DBP. 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.

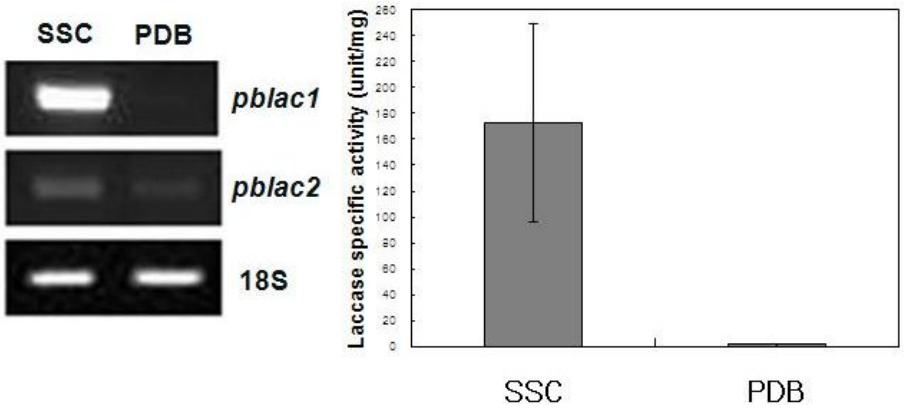

Figure 2-6. Gene expression and specific activity of laccase in *P. brumalis* mycelia cultured in different media. The RNA was extracted from 10-day-old liquid cultured *P. brumalis* mycelia in SSC medium and PDB medium. After reverse transcription was performed, as described by the kit manufacturer, 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. Specific activity of laccase was determined by the oxidation of ABTS as a substrate.

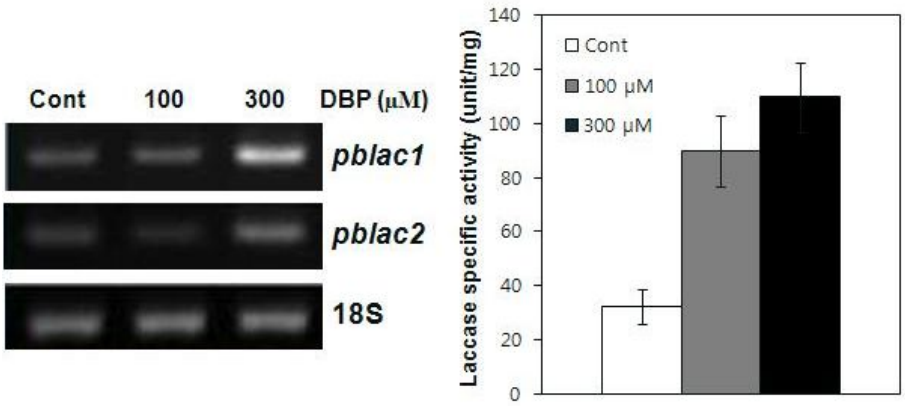

Figure 2-7. Gene expression and specific activity of laccase in *P. brumalis* mycelia in response to DBP. DBP (final conc. 100 μM and 300 μM) was applied to the liquid cultures at 5 days after pre-culture. The RNA was extracted on the 9th day after DBP treatment. Cont indicates no treatment. The expression 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. Specific activity of laccase was determined by the oxidation of ABTS as a substrate.

2.3.4. Overexpression of the pblac1 gene using a homologous genetic transfromation system in P. brumalis

In gene expression anlaysis, the transcription level of *pblac1* was higher than that of *pblac2* and was proportional to the laccase specific activity, suggesting that the transciption level of *pblac1* plays an important role in laccase activity. Thus, the cDNA of *pblac1* was used to construct strains with enhanced laccase activity using a homologous genetic transformation system. Transformants were constructed in *P. brumalis,* by the REMI method as described by Leem et al. (1999) with slight modifications. The phosphinothricin resistance gene was used as a selectable maker in the previous research, but *P. brumalis* mycelium showed stable resistance against the antibiotic phosphinothricin. For this reson, a new selectable maker was needed for transformation of *P. brumalis*. Hygromycin B, which has been frequently used as a selectable maker in plant transformation systems, inhibited growth of *P. brumalis* mycelium (Figure 2-8). The concentration of 50 μg mL^{-1} hygromycin B completely inhibited growth of mycelium.

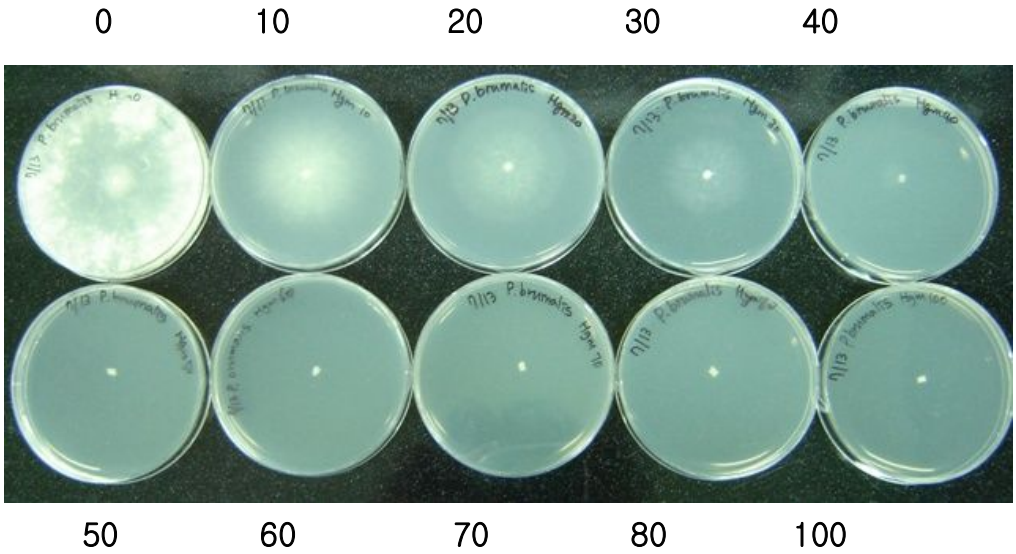

Figure 2-8. Mycelial growth inhibition of *P. brumalis* by hygromycin B. Values were expressed the concentration of hygromycin B from 0 μg mL^{-1} to 100 μg mL^{-1}).

The transformants were selected from minimal medium plus 50 μg mL^{-1} hygromycin B, and transferred to new medium containing higher concentrations hygromycin B (100 μg mL^{-1}) to examine their hygromycin resistance. To assess the production of laccase by the transformants, the absorbance of *o*-tolidine oxidation by laccase was measured using a spectrophotometer at 590 nm.

The genomic integration of the laccase-expressing vector (pHYlac1)

was confirmed by *hph*-specific PCR, and the expected amplified band appeared only in the transformants (T21 and T26) (Figure 2-9).

Increases in the absorbencies by the oxidation of *o*-tolidine showed laccase activity indirectly. The two transformants showed laccase activity approximately 3-4 times higher than that of the wild type strain in the liquid medium (Figure 2-10). This result is likely due to the fact that the glyceraldehydes-3-phosphate dehydrogenase gene (*gpdA*) promoter of *Aspergillus nidulans* (Punt et al., 1992) was used to overexpress the *pblac1* in the pHYlac vector. A homologous promoter for transformation was used in a few white-rot fungi including *T. versicolor* and *L. edodes*. However, no information about promoter sequences in *P. brumalis* has been reported, which limited the development of a homologous expression system for transformation of *P. brumalis*. In this study, the *gpd* promoter of *A. nidulans* was used for overexpression of *pblac1* in *P. brumalis* since the promoter was widely used to direct expression of target genes constitutively in fungi. When *P. tremellosa* and *T. versicolor* were transformed with the MnP cDNA driven by the *gpd* promoter, the transformants showed higher MnP activity (Yeo et al., 2007; Kum et al., 2009). Although the properties of the recombinant protein, such as its molecular weight and specific

laccase activity, were not yet confirmed, the yield of laccase from the transformants with a cloned gene was elevated, and is expected to contribute to the utilization of bioremediation.

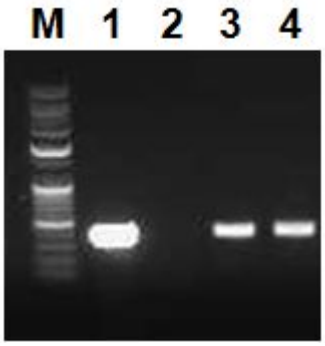

Figure 2-9. Confirmation of genomic integration of the expression vector into the host chromosomal DNA by RT-PCR in the wild type and transformants of *P. brumalis* (M, molecular weight maker; 1, vector plasmid; 2, wild type strain; 3 and 4, transformant).

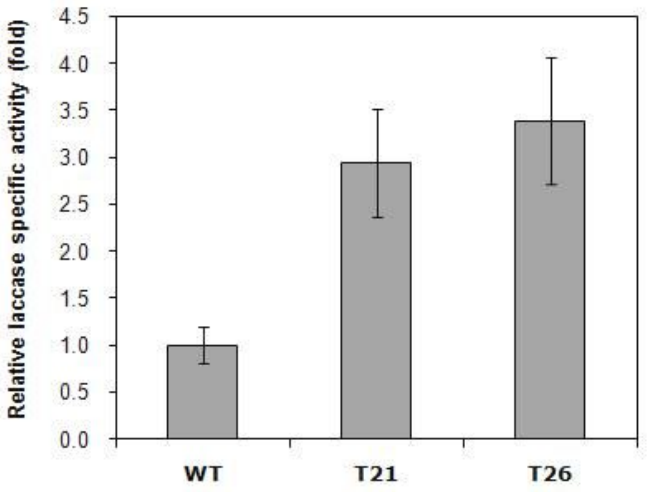

Figure 2-10. Analysis of laccase activity in culture broth of the wild type (WT) and 2 transformed strains (T21 and T26) of *P. brumalis*. The values represent the mean ± SE of 3 measurements with 3 replicates.

2.3.5. Enhanced decolorization ability of transformants

Laccases are involved in the degradation of diverse recalcitrant compounds (e.g., dye) (Pazarlioglu, et al., 2010; Sathishkumar et al., 2010; Saşmaz et al., 2011). In order to investigate the effect of laccase overexpression in *P. brumalis*, the decolorization activity of the transformants was examined using an anthraquinone RBBR dye. As shown in Figure 2-11, the transformants exhibited higher decolorization capabilities than the wild type. The transformants, T21 and T26,

decolorized 90-93% of the dye while the wild type decolorized much less than the transformants (<21%), coincidently with the laccase activity (Figure 2-11). Purified *Laccase 1* decolorized RBBR by 70% without a mediator (Kim et al., 2012), which was encoded by the same gene used to generate the pHYlac1 constructed. Thus, the results in Figure 2-10 and 2-11 suggested that the laccase activity of the transformants directly correlates with the decolorization of the dye. Laccases are multicopper blue oxidases and catalyze the oxidation of a wide range of inorganic and aromatic substances by the removal of electrons and the simultaneous reduction of O_2 to water (Xu, 1996). Due to their oxidation power and relatively low specificity, laccases can oxidize various chemical structures (Husain, 2006). Several studies reported correlations between the decolorization of dyes and the ligninolytic ability of several fungal strains (Wunch et al., 1997; Yang 2011; Grinhut et al., 2011). Therefore, the decolorization of RBBR was used to detect ligninolytic activity of fungi (Glenn and Gold, 1983; Pasti and Crawford, 1991). The ligninolytic enzymes such as laccase decolorize the RBBR as a result of a redox reaction (Mechichi et al., 2006). Thus, the data in Figure 2-11 indicate that the transformants have increased lignin-degradation activity.

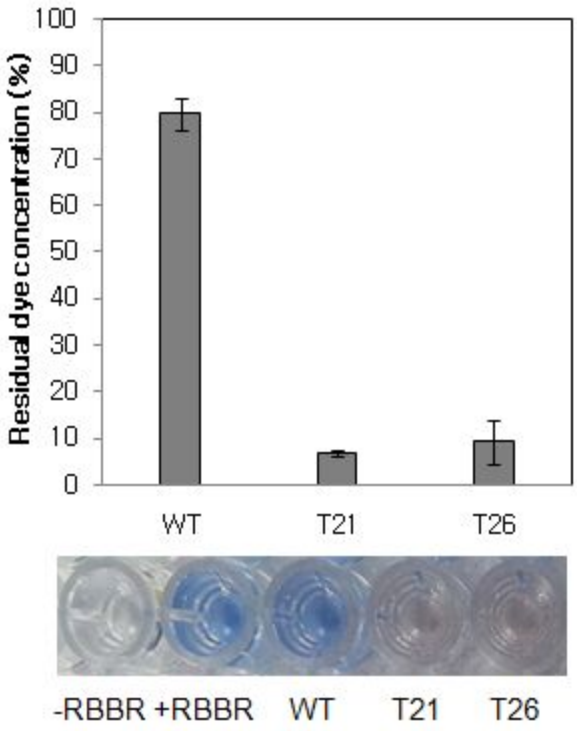

Figure 2-11. Decolorization of RBBR dye in culture broth of the wild type (WT) and 2 transformed strains (T21 and T26) of *P. brumalis*. The values represent the mean ± SE of 3 measurements with 3 replicates.

2.4. Conclusions

Two laccase cDNAs, *pblac1* and *pblac2*, were isolated from *P. brumalis*. They showed 70% similarity in deduced amino acid sequences but genomic structure and transcript levels were different each other. An RT-PCR analysis revealed that the transcript level of *pblac1* was higher than that of *pblac2* and increased by DBP treatment. The overexpression of the *pblac1* gene was derived by the promoter of a gene for glyceraldehyde-3-phosphate dehydrogenises (*gpd*) using a homologous transformation system in *P. brumalis*. The laccase activities and dye decolorization in the transformants were significantly higher than that of the wild type. The identification of cDNAs of laccases was the first step to characterize molecular events related to an ability of *P. brumalis* in the degradations of lignin as well as many recalcitrant xenobiotics. A homologous transformation system of *P. brumalis* was useful for generation of enhanced laccase activity strains.

CHAPTER III

Molecular characterization of manganese peroxidases from white-rot fungus, *Polyporus brumalis*

3.1. Introduction

White-rot fungi degrade lignin and a broad range of diverse aromatic pollutants using oxidative extracellular enzymes; includinglignin peroxidase (LiP), manganese lignin peroxidase (MnP), and laccase. MnP (EC 1.11.1.7) has been identified as the major degradative enzyme and is the most common lignin-modifying peroxidase in almost all wood-colonizing basidiomycetes. MnPs are extracellular glycosylated heme-containing enzymes that catalyze the H_2O_2-dependent oxidation of Mn(II) to Mn(III), which mediates oxidation of a variety of phenolic substrates (Bumpus et al., 1985; Paice et al., 1993; Urzúa et al., 1995). MnPs have potential applications in treating environmental pollutants (Sedighi et al., 2009).

Isozymes of MnP in *P. chrysosporium* are known to play an important role in initiating the lignin degrading reaction where they cleave lignin structures extracellularly during the first step of lignin mineralization (Gold et al., 1984; Tien and Kirk 1984). MnP from *T. versicolor* has been shown to be active in delignification of kraft pulp, decolorization of bleach plant effluents, and degradation of several dyes (Gill et al., 2002; Pasti-Grigsby et al., 1992).

MnPs are often produced in multiple forms; up to 11 different

isoforms have been described in one fungal strain (Lobos et al., 1994; Urzua et al., 1995). The complexity of the physiological processes in which these isoenzymes are involved complicates an understanding of the specific function of each of these enzymes *in vivo* and their specific roles in the degradation of lignin and various xenobiotics.

Thus far, MnPs have been isolated, by genome analysis and molecular cloning, from various fungi including *P. chrysosporium* (Tien and Tu 1987**;** Pribnow et al., 1989; Dass and Reddy 1990; Orth et al., 1994), *P. ostreatus* (Kamitsuji et al., 2004), *T. versicolor* (Johansson et al., 2002), and others (Manubens et al., 2003; Hakala et al., 2006). The MnP isozymes are encoded by multiple genes (Pribnow et al., 1989; Pease and Tien 1992). *P. chrysosporium* has four MnP isozymes, viz., H3, H4, H5, and H9 (Kirk et al., 1984) and the three distinct *mnp* genes have been isolated and sequenced: *mnp-1* encodes H4; *mnp-2* encodes an unidentified protein product, and *mnp-3* encodes H3 (Pribnow et al., 1989 Orth et al., 1994). Analysis of the recently sequenced genome of *P. chrysosporium* RP78 identified five MnP genes (Martinez et al., 2004). *T. versicolor* has at least four MnP isogenes (Kim et al., 2005; Yeo et al., 2007). They showed high lignin-degrading activity (Johansson et al., 2002) and the capacity to degrade

various phenanthrene compounds (Han et al., 2004). However, the specific functions of each of these isoforms in relation to degradation of lignin or other chemical compounds remain to be determined.

In this chapter, six MnP were cloned from *P. brumalis* mycelia, and their expression patterns were discribed in response to culture conditions; an efficient homologous expression system for overexpression of MnP was constructed in *P. brumalis* mycelia.

3.2. Materials and Methods

3.2.1. Cultivation and treatment conditions

P. brumalis KFRI 20912 was maintained on a potato dextrose agar (PDA) plate and grown in 300 mL of liquid potato dextrose broth (PDB) or shallow stationary culture (SSC) liquid medium at 28°C. *P. brumalis* was pre-grown in SSC liquid medium for 5 days at 28°C and then MnP was induced with 100 or 300 μM DBP. The induced culture was incubated for additional 9 days. For degradation of lignin in the wood chip, three pieces of wood chip (7×3×1.5 mm) were placed onto the surface of PDA agar covered with fungal mycelia and were then incubated for 40 days. Total RNA was extracted from mycelia covering the wood chip at the 20^{th} and 40^{th} day after inoculation. Changes in the mRNA levels were compared to the control sample, which had been cultured on PDA medium in the absence of wood chips.

3.2.2. Analysis of DNA and protein sequences

Two degenerated primers were designed for cloning of partial MnP cDNAs: a forward primer (F1); 5′-CACGACGCCATCGSCATCTC-3′ and a reverse primer (R1); 5′-GTGCGASRCSAGMAGSGCAAC-3′, which corresponded to the metal-binding regions of the previously

reported MnP sequences at the National Center for Biotechnology Information; Tv-mnp (*T. versicolor*): BAB03464, Pr-mnp (*Phlebia radiata*): CAC84573, and Pc-mnp1 (*P. chrysosporium*): AAA33744 (Godfrey et al., 1990; Hilden et al., 2009). RT-PCR was performed as described in Section 2.2.3. Full-length cDNAs of the *pbmnp* genes were amplified by RT-PCR with 5′-and 3′-primers designed according to the nucleotide sequence data obtained from rapid amplification of cDNA ends (RACE)-products.

The sequences of the full length cDNA have been deposited in GenBank of NCBI under the accession numbers HQ910411, HQ910412, HQ910413, HQ910414, HQ910415, and HQ910416 for *pbmnp1-6*, respectively. The obtained nucleotide sequences and homologous sequences were analyzed by database searches using the BLAST search program on the NCBI web-server. To predict the molecular mass of and signal peptides in the deduced protein, ExPasy program (http://www.expasy.org/tools) was used. Sequence alignment was constructed with the ClustalX program (http://www.clustal.org).

3.2.3. Assay of MnP activity

MnP activity was determined by assessing the oxidation of 2, 6-

dimethoxyphenol (2, 6-DMP, SIGMA, MO, USA) as a substrate along with H_2O_2. The culture broth was clarified by centrifugation at 8000×*g* for 10 min. The reaction mixture contained 2 mM 2, 6-DMP, 2.5 mM $MnSO_4$, 50 mM malonate buffer (pH 4.5), 2 mM H_2O_2, and 30 μL culture supernatant in a total volume of 300 μL. Oxidation was followed by an absorbance increase at 477 nm. The Bradford method with 1 mg mL^{-1} of bovine serum albumin (BSA; Sigma, MO, USA) used as a standard (Bradford, 1976) was used to determine protein concentrations.

3.2.4. Determination of gene expression

An RT kit (Promega, Madison, WI, USA) was used to analyze mRNA expression. Reverse transcription of 2 μg aliquots of total RNA was carried out as recommended by the manufacturer. An 18S rRNA (Quantum RNA 18S internal standards kit; Ambion, Carlsbad, CA, USA) was used as internal control according to the manufacture's protocol. Conventional PCR was carried out for 0.2 μL samples of the first-strand cDNA (corresponding to approximately 20-40 ng of RNA) by adding 1.5 μL of 5 mM $MgCl_2$, 5 μL of 10×PCR buffer, 1 μL of 10 mM dNTP mix, 1 μL of sense primer (10 pmol), 1 μL of antisense

primer (10 pmol), 1 U of Taq DNA polymerase, and water to a final volume of 20 μL. PCR amplification reactions were initially incubated at 94°C for 5 min, followed by 30-35 cycles at 94°C for 30 s, 42-58°C (depending on the melting temperature of each set of gene-specific primers) for 30 s, and 72°C for 30 s. Reaction products (20 μL) were analyzed by gel electrophoresis.

Gene-specific primers for PCRs were designed from the 3′-UTRs or the region near the translation stop codon of each gene. The primer sets were 5′-CGTTCCCAATGCATGATCAC-3′ and 5′-TACACCGTCCGCCACAAC-3′ for *pbmnp1*, 5′-TGACCAACGATGACCTTC-3′ and 5′-GTGTAAGTCGCCTTTTTCAG-3′ for *pbmnp2*, 5′-GTGTCAGACCGTGAAGCG-3′ and 5′-ACTGACAATCTGACTCTCTACGA-3′ for *pbmnp3*, 5′-CTCGCTCGTGACGACTCTCG-3′ and 5′-ACATAATCTCAATGATAGACCAG-3′ for *pbmnp4*, 5′-AACCTATAAGCCGTTCACC-3′ and 5′-TTCGCTGTAGGGAACAT-3′) for *pbmnp5*, and 5′-TCCTTCCTAAATATTCACCG-3′ and 5′-GTTGGATACGGGTTTACTCA-3′ for *pbmnp6*. The nucleotide

sequences of RT-PCR products were identical to those of the corresponding MnP cDNAs.

3.2.5. Transformation and expression of MnP gene in P. brumalis

A phmnp4-expressing vector (pHYmnp4) was constructed by modifying pBARGPE1 as descrbed in Chapter 2. The *pbmnp4* cDNA was inserted between the *Bam*HI and *Xho*I sites of the modified vector (Figure 3-1).

P. brumalis was transformed with pHYmnp4 (5 μg) by the REMI method, as described in Chpater II. Genomic DNAs were extracted from the wild type and transformants were used as the template for PCR. PCR was performed using two primers specific for *hph*: 5'-TGGATATGTCCTGCGGGTAA-3', 5'-CGTCAGGACATTGTTGGAGC-3'.

MnP activities of the wild type and the transformants were analyzed using 2, 6-DMP. The MnP gene expression levels of the transformants that showed higher enzyme activity than the wild type were determined by qRT-PCR using two primers specific for *pbmnp4*. Total RNAs were isolated from the 14 day-old mycelium. To evaluate the decolorization of Remazol Brilliant Blue R (RBBR), the assay system (300 μL volume) contained a filtrate of crude broth, obtained after 6 days'

incubation of each transformant and wild type strains in liquid culture in SSC media, 100 μg mL^{-1} RBBR, and 100 nmol mL^{-1} H_2O_2. After 5 h incubation at room temperature, the absorption of residual RBBR at 590 nm was used as a measure of the decolorization activity.

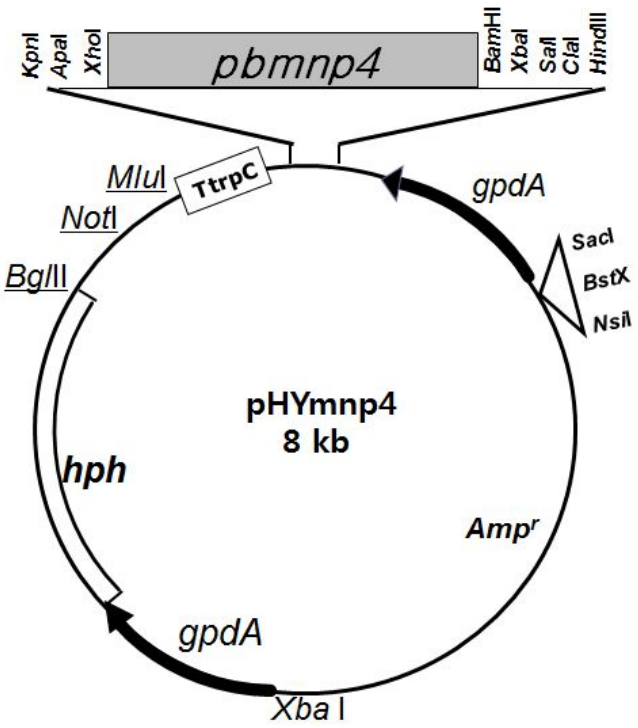

Figure 3-1. Restriction map of the transforming plasmid pHYmnp4. The sequence encoding laccase gene, *pbmnp4,* is under the control of the promoter of the *gpd* gene (*gpdA*). A hygromycine resistance gene was indicated as *hph*.

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3.3. Results and Discussion

3.3.1. Isolation and sequence determination of MnP cDNAs

MnP cDNA fragments were isolated from the total RNA of *P. brumalis* by RT-PCR using degenerate oligonucleotides corresponding to the metal-binding regions of MnP. The full length cDNAs were cloned using RACE. The characteristics of the six cDNAs are listed in Table 3-1. Among the MnP-encoding genes, *pbmnp4* was the largest. The 5'-UTR of *pbmnp4* mRNA was 764 nucleotides, which was longer than that of any other MnP genes; moreover, it contained two additional ATG start codons at -219 and -171 from the conventional start codon. It is unclear whether all start codons in *pbmnp4* are in fact used, but a variety of polypeptides with different N-terminal sequences could be produced if the different start codons were used, similar to what has been observed for human phosphodiesterase 9A (Carles and Pere 2006). Another possibility is that the long 5'-UTR functions as a translational enhancer (Nguyen 2009). It was noted, however, that the deduced MnP protein sequences were of similar sizes, i.e., 360-365 amino acids long.

Comparison of the deduced amino acid sequences revealed that these six MnPs share 203 conserved residues across the highly conserved metal-binding regions in Figure 3-2 (Canales et al., 1998). The Mn^{2+}-

binding sites consisted of the three acidic residues (indicated by arrows in Figure 3-2) for the internal heme propionate to oxidize Mn^{2+} (Sundaramoorthy et al., 1997). Additional Mn-binding residues which are involved in the oxidation of aromatic substrates via long-range electron transfer pathways (Schoemaker et al., 1994; Choinowski et al., 1999) are located on the protein surface (filled diamond symbols in Figure 3-2). The proteins also contained two histidine residues (empty diamonds in Figure 3-2) that are essential for catalysis. Table 3-2 gives an overview of the results of amino acid sequence comparisons between the products of these cDNAs. They show 62-96% amino acid identities including a number of that are obviously conserved for structural reasons, such as the metal binding regions.

Table 3-1. Relevant features of sequences of the six MnP cDNAs isolated from mycelia of *P. brumalis*

Sequence features	cDNA					
	pbmnp1	*pbmnp2*	*pbmnp3*	*pbmnp4*	*pbmnp5*	*pbmnp6*
Length of cDNA (bp)	1,269	1,274	1,410	2,011	1,471	1,300
5' UTR (bp)	17	90	95	764	211	77
3' UTR (bp)	154	101	167	152	193	128
Length of predicted mature protein (aa)	365	360	363	364	365	364
Molecular weight of predicted mature protein (kDa)	38.92	38.48	38.02	38.10	39.02	37.99
pI of predicted mature protein	4.26	4.48	4.25	4.25	4.54	4.07
Target site	Secreted	Secreted	Secreted	Secreted	Secreted	Secreted

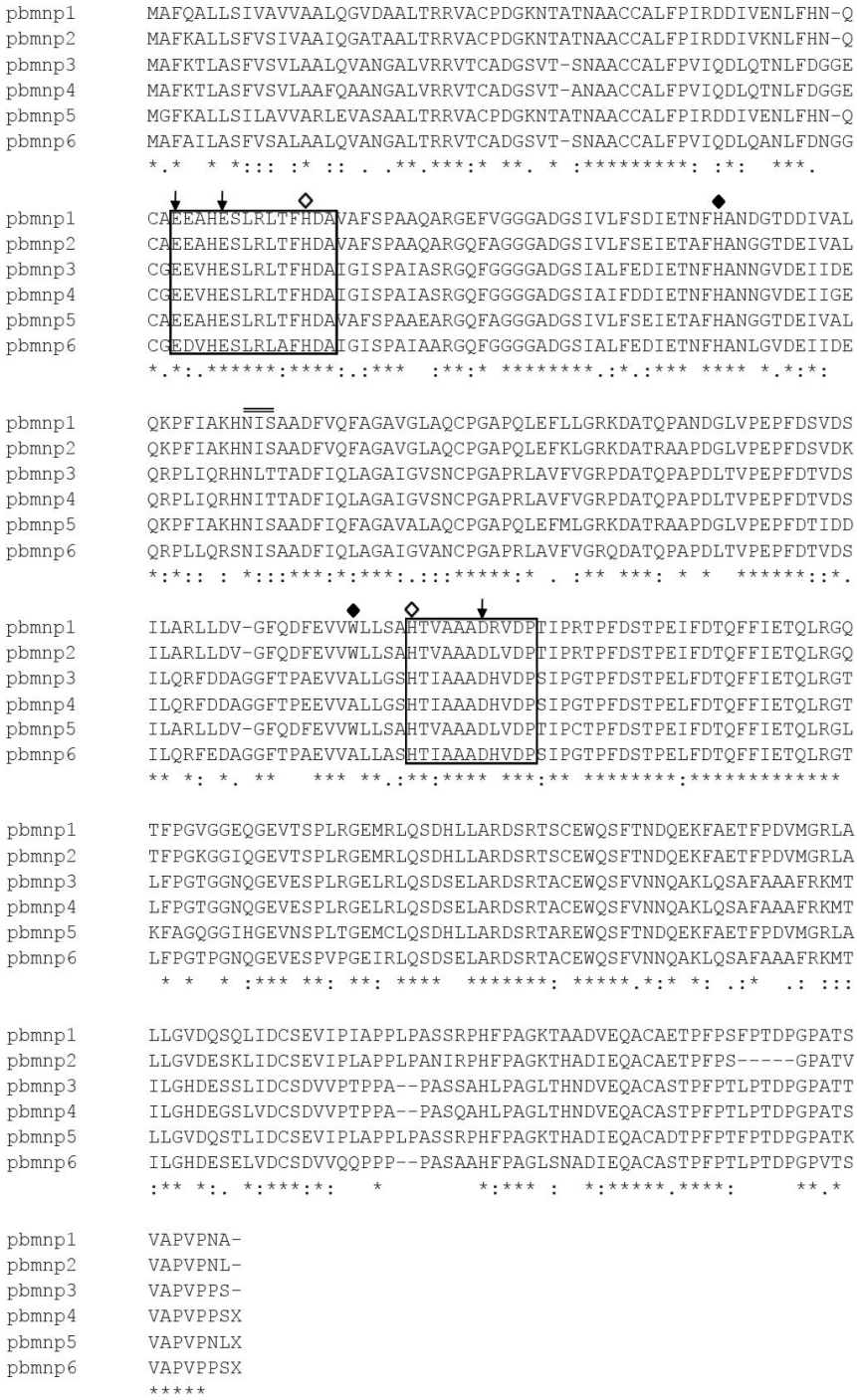
pbmnp1 MAFQALLSIVAVVAALQGVDAALTRRVACPDGKNTATNAACCALFPIRDDIVENLFHN-Q
pbmnp2 MAFKALLSFVSIVAAIQGATAALTRRVACPDGKNTATNAACCALFPIRDDIVKNLFHN-Q
pbmnp3 MAFKTLASFVSVLAALQVANGALVRRVTCADGSVT-SNAACCALFPVIQDLQTNLFDGGE
pbmnp4 MAFKTLASFVSVLAAFQAANGALVRRVTCADGSVT-ANAACCALFPVIQDLQTNLFDGGE
pbmnp5 MGFKALLSILAVVARLEVASAALTRRVACPDGKNTATNAACCALFPIRDDIVENLFHN-Q
pbmnp6 MAFAILASFVSALAALQVANGALTRRVTCADGSVT-SNAACCALFPVIQDLQANLFDNGG
pbmnp1 CAEEAHESLRLTFHDAVAFSPAAQARGEFVGGGADGSIVLFSDIETNFHANDGTDDIVAL
pbmnp2 CAEEAHESLRLTFHDAVAFSPAAQARGQFAGGGADGSIVLFSEIETAFHANGGTDEIVAL
pbmnp3 CGEEVHESLRLTFHDAIGISPAIASRGQFGGGGADGSIALFEDIETNFHANNGVDEIIDE
pbmnp4 CGEEVHESLRLTFHDAIGISPAIASRGQFGGGGADGSIAIFDDIETNFHANNGVDEIIGE
pbmnp5 CAEEAHESLRLTFHDAVAFSPAAEARGQFAGGGADGSIVLFSEIETAFHANGGTDEIVAL
pbmnp6 CGEDVHESLRLAFHDAIGISPAIAARGQFGGGGADGSIALFEDIETNFHANLGVDEIIDE
pbmnp1 QKPFIAKHNISAADFVQFAGAVGLAQCPGAPQLEFLLGRKDATQPANDGLVPEPFDSVDS
pbmnp2 QKPFIAKHNISAADFVQFAGAVGLAQCPGAPQLEFKLGRKDATRAAPDGLVPEPFDSVDK
pbmnp3 QRPLIQRHNLTTADFIQLAGAIGVSNCPGAPRLAVFVGRPDATQPAPDLTVPEPFDTVDS
pbmnp4 QRPLIQRHNITTADFIQLAGAIGVSNCPGAPRLAVFVGRPDATQPAPDLTVPEPFDTVDS
pbmnp5 QKPFIAKHNISAADFIQFAGAVALAQCPGAPQLEFMLGRKDATRAAPDGLVPEPFDTIDD
pbmnp6 QRPLLQRSNISAADFIQLAGAIGVANCPGAPRLAVFVGRQDATQPAPDLTVPEPFDTVDS
pbmnp1 ILARLLDV-GFQDFEVVWLLSAHTVAAADRVDPTIPRTPFDSTPEIFDTQFFIETQLRGQ
pbmnp2 ILARLLDV-GFQDFEVVWLLSAHTVAAADLVDPTIPRTPFDSTPEIFDTQFFIETQLRGQ
pbmnp3 ILQRFDDAGGFTPAEVVALLGSHTIAAADHVDPSIPGTPFDSTPELFDTQFFIETQLRGT
pbmnp4 ILQRFDDAGGFTPEEVVALLGSHTIAAADHVDPSIPGTPFDSTPELFDTQFFIETQLRGT
pbmnp5 ILARLLDV-GFQDFEVVWLLSAHTVAAADLVDPTIPCTPFDSTPEIFDTQFFIETQLRGL
pbmnp6 ILQRFEDAGGFTPAEVVALLASHTIAAADHVDPSIPGTPFDSTPELFDTQFFIETQLRGT
pbmnp1 TFPGVGGEQGEVTSPLRGEMRLQSDHLLARDSRTSCEWQSFTNDQEKFAETFPDVMGRLA
pbmnp2 TFPGKGGIQGEVTSPLRGEMRLQSDHLLARDSRTSCEWQSFTNDQEKFAETFPDVMGRLA
pbmnp3 LFPGTGGNQGEVESPLRGELRLQSDSELARDSRTACEWQSFVNNQAKLQSAFAAAFRKMT
pbmnp4 LFPGTGGNQGEVESPLRGELRLQSDSELARDSRTACEWQSFVNNQAKLQSAFAAAFRKMT
pbmnp5 KFAGQGGIHGEVNSPLTGEMCLQSDHLLARDSRTAREWQSFTNDQEKFAETFPDVMGRLA
pbmnp6 LFPGTPGNQGEVESPVPGEIRLQSDSELARDSRTACEWQSFVNNQAKLQSAFAAAFRKMT
pbmnp1 LLGVDQSQLIDCSEVIPIAPPLPASSRPHFPAGKTAADVEQACAETPFPSFPTDPGPATS
pbmnp2 LLGVDESKLIDCSEVIPLAPPLPANIRPHFPAGKTHADIEQACAETPFPS-----GPATV
pbmnp3 ILGHDESSLIDCSDVVPTPPA--PASSAHLPAGLTHNDVEQACASTPFPTLPTDPGPATT
pbmnp4 ILGHDEGSLVDCSDVVPTPPA--PASQAHLPAGLTHNDVEQACASTPFPTLPTDPGPATS
pbmnp5 LLGVDQSTLIDCSEVIPLAPPLPASSRPHFPAGKTHADIEQACADTPFPTFPTDPGPATK
pbmnp6 ILGHDESELVDCSDVVQQPPP--PASAAHFPAGLSNADIEQACASTPFPTLPTDPGPVTS
pbmnp1 VAPVPNA-
pbmnp2 VAPVPNL-
pbmnp3 VAPVPPS-
pbmnp4 VAPVPPSX
pbmnp5 VAPVPNLX
pbmnp6 VAPVPPSX

Figure 3-2. Alignment of the predicted amino acid sequences of the six MnPs from *P. brumalis*. The conserved residues are indicated by asterisks. The boxes mark the conserved Mn^{2+}-binding domain and arrows indicate the Mn-binding site enabling oxidation of Mn^{2+}. The diamonds indicate residues that are involved in oxidation of aromatic substrates. The two histidines (H) that are essential for catalysis are indicated by unfilled diamonds. *N*-glycosylation site is indicated by a double line. GenBank accession nos. for *pbmnp1*, *pbmnp2*, *pbmnp3*, *pbmnp4*, *pbmnp5*, and *pbmnp6* are HQ910411, HQ910412, HQ910413, HQ910414, HQ910415, and HQ910416.

Table 3-2. Pairwise comparison of the six MnPs encoded by cDNAs isolated from mycelia of *P. brumalis*

Protein[a]	*pbmnp1*	*pbmnp2*	*pbmnp3*	*pbmnp4*	*pbmnp5*	*pbmnp6*
pbmnp2	89	-	-	-	-	-
pbmnp3	64	63	-	-	-	-
pbmnp4	63	62	96	-	-	-
pbmnp5	88	89	63	62	-	-
pbmnp6	63	62	90	88	62	-

[a] The values indicate the degree (%) of amino acid sequence identity between the six MnPs.

3.3.2. Genomic organization of the six MnP genes

To elucidate the genomic organization of the six MnP genes, 3'-UTRs of each gene were amplified from cDNAs, cloned to a pGEM-T easy vector, sequenced and used as probes. Southern analysis of the *Pst*I-, *Eco*RV-, *Bam*HI- and *Xho*I-digested genomic DNA is shown in Figure 3-3. The hybridization bands detected by gene-specific probes differed in size and number, suggesting that the genes corresponding to six MnPs are not clustered in the genome and have different intron-exon structures. The families of secreted heme enzymes, MnPs contain six or seven short introns that the sequences surrounding active-site residues are conserved (Gold et al., 1993). Even though MnPs have the conserved site, they are shown in different size and number. The pattern of intron distribution for isozyme genes depends on species. The *P. chrysosporium* MnP genes have a conserved exon-intron pattern but 3 MnP genes *Fomitiporia mediterranea* have different positions of intron each other (Morgenstern et al., 2010). MnP isoforms are differently regulated by carbon and nitrogen sources (Gettemy et al., 1998). They is also regulated at the level of gene transcription by Mn(II) or Mn(III) ligands, the substrate for the enzyme and by heat shock (Gettemy et al., 1998; Morgenstern et al., 2010). Therefore, it is presumed that MnP

isoforms have different *cis*-elements and structures on the upstream promoter region of each gene and it will be needed to study about the regulation mechanisms of the region from *P. brumails*.

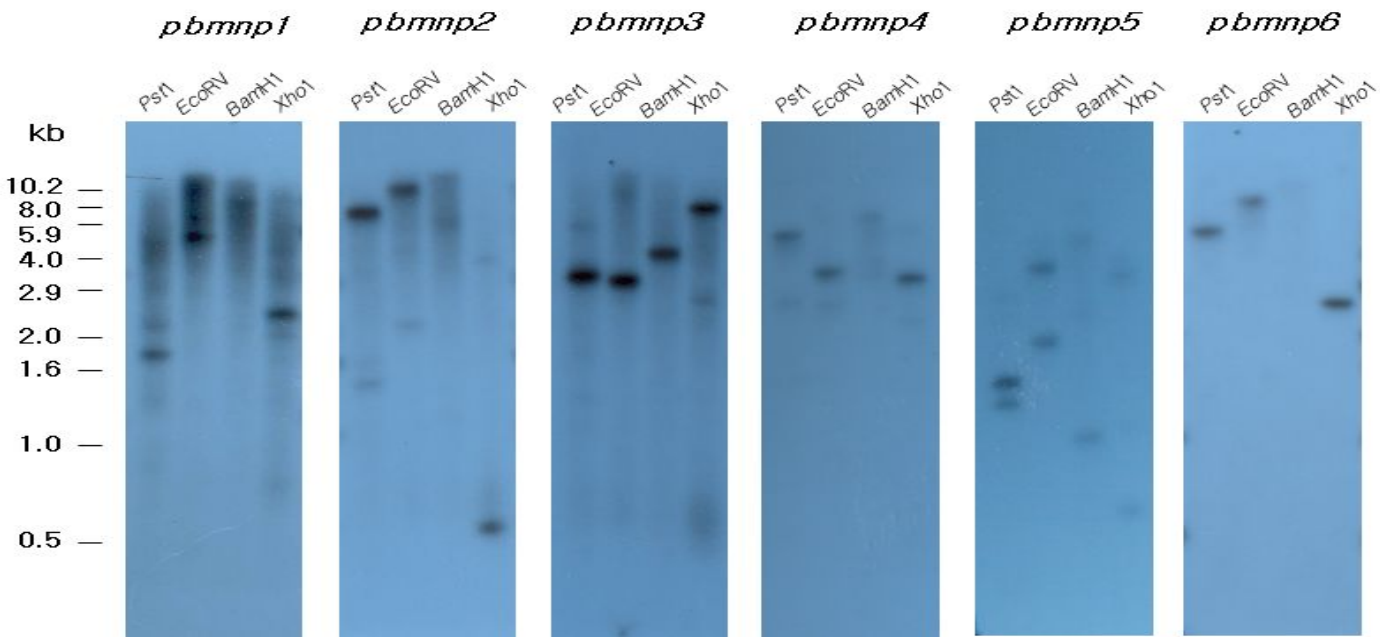

Figure 3-3. Genomic Southern analysis of the MnP genes from *P. brumalis*. Equal amounts (15 μg) of DNA were digested with the restriction enzymes, fractionated by electrophoresis, transferred onto a nylon membrane and probed with ^{32}P-labeled gene-specific DNA fragments.

3.3.2. Gene expression analysis

To investigate the effects of medium compositions on the expression of the MnP genes, RT-PCR analysis was conducted with mycelia cultured in PDB or SSC media. As shown in Figure 3-4, the MnP genes were predominantly expressed in the SSC medium, although the expression level of each MnP gene was different. This coincided with the MnP activity.

P. brumalis has been reported to degrade DBP efficiently using a ligninolytic enzyme system (Lee et al., 2005; Lee et al., 2007a). As shown in Figure 3-5, the gene expression levels and activities of MnP increased with the increase in DBP concentrations. In particular, *pbmnp4* expression peaked in the presence of 300 μM DBP. Both *pbmnp2* and *pbmnp5* were expressed at a low level when *P. brumalis* was treated with 100 μM DBP, but were strongly induced by treatment with 300 μM DBP. The expression of *pbmnp1*, *pbmnp3*, and *pbmnp6* increased moderately in the presence of DBP.

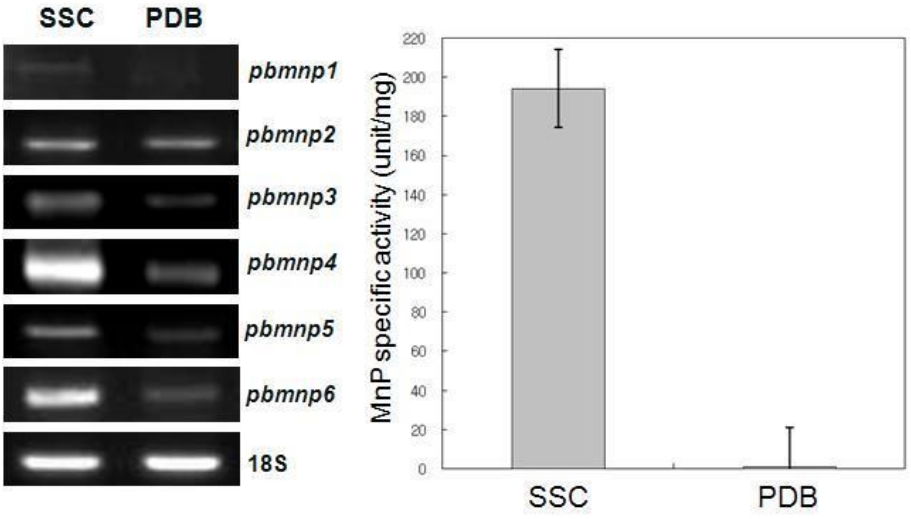

Figure 3-4. Gene expression and specific activity of MnP in *P. brumalis* mycelia cultured in different media. RNA was extracted from 10-day-old liquid cultures of *P. brumalis* mycelia in SSC and PDB medium.

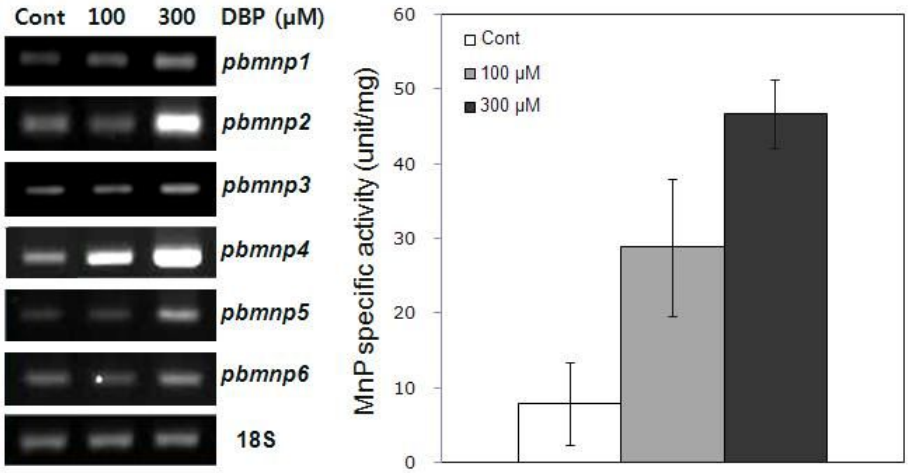

Figure 3-5. Gene expression and specific activity of MnP in *P. brumalis* mycelia in response to DBP. DBP (final concentration: 100 μM and 300 μM) was applied to liquid cultures at 5 days after pre-culture. RNA was extracted at the 9th day after treatment with DBP. Relative intensity of gene expression was normalized against the amount of 18S rRNA as the endogenous reference gene.

To elucidate the significance of MnP for fungal growth on natural lignocelluloses, MnP gene expression was investigated while growing *P. brumalis* on wood chips. RNA was extracted from mycelia covering wood chips at the 20^{th} and 40^{th} day after inoculation. The amount of each type of transcript was normalized against the amount of 18S rRNA as the endogenous reference gene, and was compared to the control sample that had been cultured on PDA medium only for 20 or 40 days (Figure 3-6). Most of the MnP genes were up-regulated in parallel with an increase in the corresponding enzyme activities: An increase in mRNA expression by the 40^{th} day was statistically significant for *pbmnp4* and *pbmnp6*.

3.3.3. Overexpression of pbmnp4 using a homologous expression system in P. brumalis

The transcription level of *pbmnp4* was proportional to the MnP specific activity in response to DBP and wood chip, which indicated that the transciption level of *pbmnp4* plays an important role in MnP activity. In order to construct recombinant *P. brumalis* with high MnP activity, an expression vector facilitating overexpression of *pbmnp4*

under the control of the constitutive promoter (*gpdA*) was constructed and introduced into *P. brumalis*. The transformants of *pbmnp4* were grown in minimal medium containing hygromycin B. PCR using primers specific for the constructed plasmid analyzed the integration of the gene constructs in the transformants. To assess production of MnP in the transformants, MnP activity was measured by the DMP-oxidation method. Three transformants that showed higher MnP activities were selected for further experiments. The MnP activity in the transformants was 2-12 times higher than that of the wild type strain (Figure 3-7). This difference may arise from different numbers of integrated copies. The *pbmnp4* gene transcription levels of the three transformants were compared with that of the wild type strain by qRT-PCR. The transformants showed the increased transcription levels of the *pbmnp4* gene, indicating that the increased enzyme activity is due to the enhanced transcription levels of the *pbmnnp4* gene (Figure 3-7).

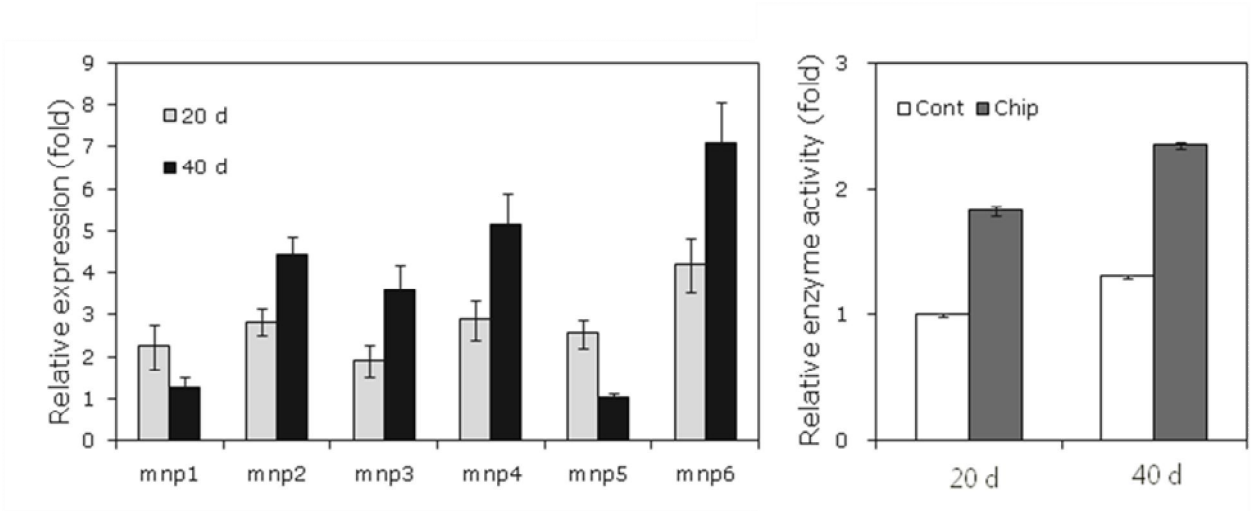

Figure 3-6. Change in gene expression and activity of MnP during cultivation with wood chips. *P. brumalis* was cultured on a chip of *P. densiflora* (7×3×1.5 mm) after pre-culture for 10 days on solid PDA medium. RNA was extracted from mycelia covering the wood chip on the 20th and 40th day after.

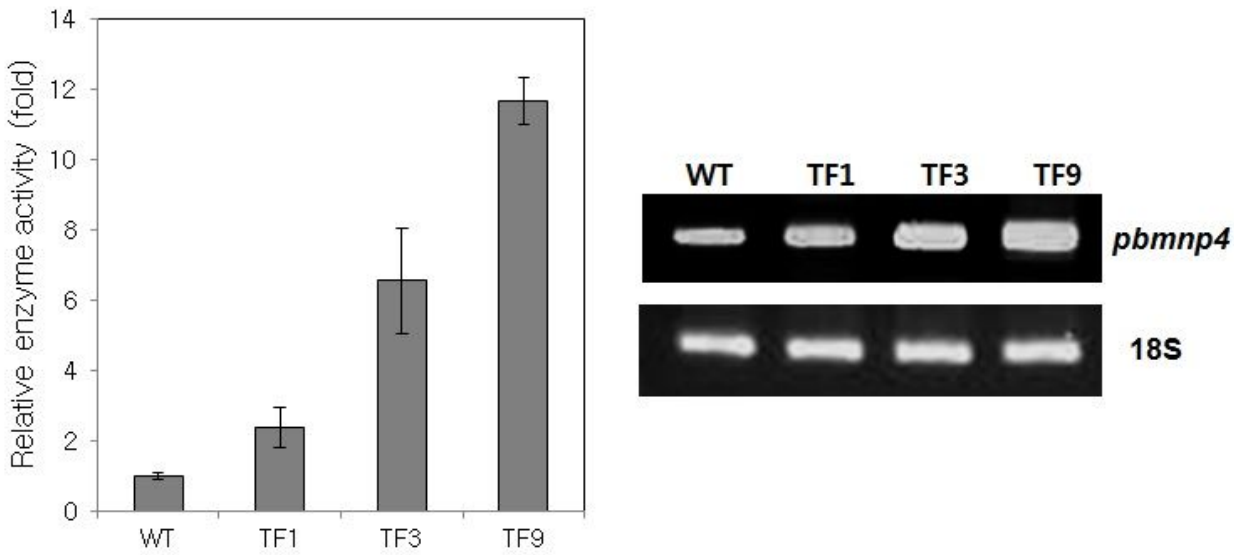

Figure 3-7. Comparison of the extracellular MnP activities and the *pbmnp4* gene expressions of the wild type and 3 transformed strains on the 14th day during culture in SSC medium. Values represent the mean ± SE of 3 measurements with 3 replicates.

3.3.4. Decolorization of RBBR by the transformants

In order to investigate the potential use of *P. brumalis* in bioremediation, the decolorization activity of the transformants was examined using Remazol Brilliant Blue R (RBBR). This dye is an antracene derivative and has been used to detect ligninolytic ability of fungi in other studies (Choinowski et al., 1999). As shown in Figure 3-8, the transformants exhibited higher decolorization activities than did the wild type. The transformant TF9 showed the highest decolorization activity, which coincided with the highest MnP activity and gene expression (Figure 3-7, 3-8). This result indicated that MnP activity in the transformants directly correlated with decolorization of the dye.

Although the biochemical properties of all six MnPs has not been analyzed yet, the MnP activity from the strains transformed with the cloned MnP gene, *pbmnp4*, was elevated; levels of MnP activity in transformants vaired, most likely due to differences in copy number of the introduced gene. These recombinant strains should be further studied for biotechnical applications including degradation of various recalcitrant xenobiotics and lignin. For instance, Kum et al. (2011) generated transformants of *P. tremellosa* using the laccase genomic DNA and MnP cDNA. The transformed strains secreted elevated

amounts of MnP and showed an increased ability to degradethe endocrine disrupting chemicals, bisphenol A and nonylphenol. Attempts to utilize these strains for pretreatment of wood biomass are currently underway.

MnPs play a key role in the biodegradation of aromatic compounds by redox reactions catalyzed by versatile enzymes. Ruiz-Dueñas et al. (2009) showed that versatile peroxidases have hybrid properties, due to the coexistence of different catalytic sites within a single protein. Thus, phenols and dyes are oxidized in the presence of acidic residues that make up the binding site of the Mn(II) oxidation system in MnPs. When the catalytically active site is exposed, the substrate is oxidized, initiating the electron transfer pathway.

It may be possible that RBBR oxidation was due to the presence of Mn(II) in SSC media and occurred by regulation of the main heme access channel. An understanding of the exact redox mechanism of peroxidases will require crystallographic, spectroscopic, directed mutagenesis and kinetic studies.

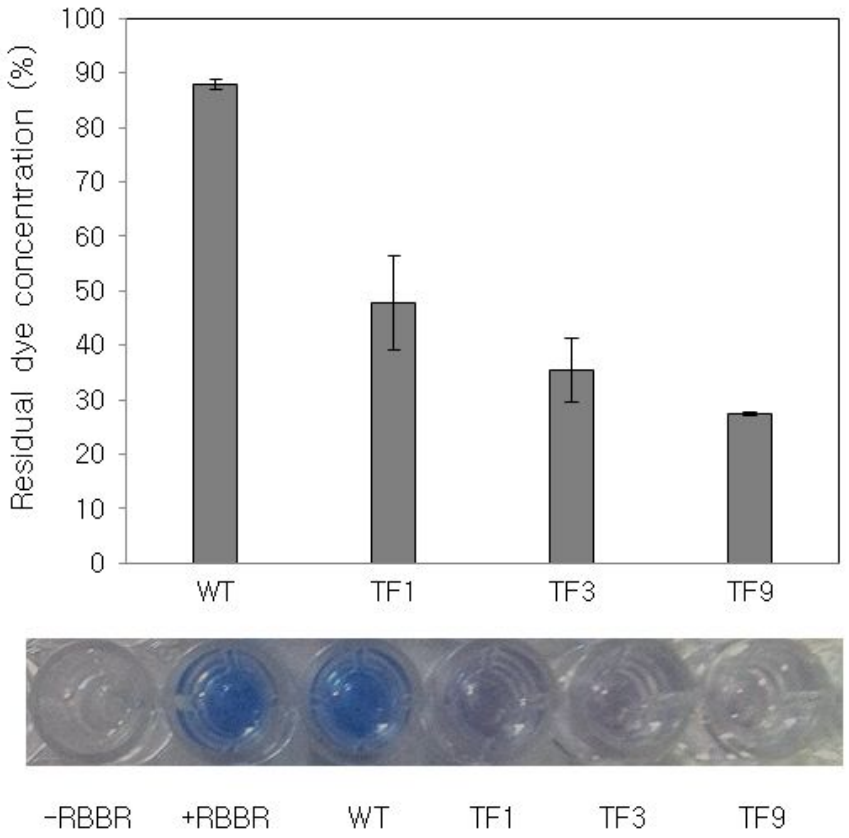

Figure 3-8. Residual concentration of RBBR after incubation with the culture broth of the wild type and 3 transformed strains. Values represent the mean ± SE of 3 measurements with 3 replicates.

3.4. Conclusions

The cDNAs of six manganese-dependent peroxidases (MnPs) were isolated from *P. brumalis*. MnP proteins share similar properties with each other in terms of size and primary structure, showing 62-96% amino acid sequence identity. Gene expression was induced by treatment with dibutyl phthalate (DBP) and wood chips. Expression of *pbmnp4* was strongly induced by the above two treatments. Overexpression of *pbmnp4* effectively increased MnP activity; the transformant that had the highest MnP activity also demonstrated the most effective decolorization of Remazol Brilliant Blue R dye. Identification of MnP cDNAs can contribute to the efficient production of lignin degradation enzymes and may lead to utilization of basidiomycetous fungi for degradation of numerous recalcitrant xenobiotics.

CHAPTER IV

Enhanced lignin biodegradation by a laccase-over expressing white-rot fungus, *Polyporus brumalis*, in the pretreatment of wood chips

4.1. Introduction

Due to the depletion of petroleum resources and the negative impact of fossil fuels on the environment (i.e., greenhouse gas emissions), there is a growing interest in the development of renewable fuels. The most common renewable fuel today is bioethanol produced mainly from sugar or starch. This resource, which is also an ingredient of animal feed and is used for a variety of human needs, is not sufficient to meet the increasing ethanol fuel demands and, as a result, grain prices have increased (Farrell et al., 2006). Therefore, lignocellulosic biomass such as those found in forests and agricultural residues may prove to be an alternative substrate for biofuel production. The first step in the conversion of lignocelluloses to biofuel is pretreatment, which is performed by degrading lignin to liberate cellulose and hemicellulose and allows for efficient enzymatic hydrolysis (Mosier et al., 2005). There has been an increasing interest in the study of microbial pretreatment of lignocelluloses (Jung et al., 2012). White-rot fungi are the most effective lignin-degrading microorganisms in nature. Biological pretreatment by white-rot fungi has previously been investigated to enhance lignocellulosic materials for paper-related applications. Recently, this environment-friendly approach has received

renewed attention as a pretreatment method for enhancing enzymatic saccharification of lignocellulosic biomass. Delignification of different lignocellulosic biomasses has been investigated for several white-rot fungi. Pretreatment of *P. ostreatus* has been shown to increase hydrolysis of biomass feedstock with a maximum glucose yield of 33% for rice straw and 38.9% for rice hull (Taniguchi et al., 2005; Yu et al., 2009b). Corn stover pretreated with *Cyathus stercoreus* showed 36% conversion of cellulose to glucose (Keller et al., 2003). Pretreatment with *E. taxodii* 2538 has been found to enhance the enzymatic hydrolysis of hardwood by a 4.7-fold (Chinese willow) and softwood (China fir) by a 6.3-fold (Yu et al., 2009a). The highest glucose yield, 66.6%, has been obtained from corn stover pretreated with *C. subvermispora* (Wan et al., 2010). However, pretreatment with these fungi is time-consuming due to their low delignification rates, which is the major barrier for large-scale applications of fungal pretreatment.

White-rot fungi can completely degrade wood lignin with ligninolytic enzymes such as laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) (Kirk et al., 1975; Kirk and Farrell, 1987). It has been reported that significant lignin degradation occurred after the production of laccase and MnP (Guerra et al., 2002; Wan and Li,

2010). Therefore, enzymes that are produced during fungal cultivation are primarily responsible for lignin depolymerization.

The laccase gene was isolated from *P. brumalis,* which exhibited a high capacity of survival under toxic conditions and have the ability to degrade recalcitrant chemicals (Lee et al., 2005; Lee et al., 2007b, Ryu et al., 2008). The recombinant *P. brumalis* was constructed by introducing a vector that overexpresses the laccase gene of this fungus. In this chapter, the recombinant *P. brumalis* strains with enhanced lignin-degradation activity were analyzed to determine the efficiency of the lignin-degradation as well as saccharification after pretreatment of lignocellulosic biomass.

4.2. Materials and Methods

4.2.1. Fungal biological pretreatment of wood chips

Each strain was pre-grown on 200 mL of PDA medium in a cultivation box for 7 days at 28°C. To examine the degradation of lignin in wood chips, *P. densiflora* and *Liriodendron tulipifera* samples were cut to obtain 70×30×4 mm portions and were sterilized at 121°C for 15 min. Three wood chips were placed onto PDA agar covered with the fungal mycelia and were then incubated for 15 and 45 days. All the cultures were grown in triplicate. A set of sterilized non-inoculated wood chips served as the control wood sample.

4.2.2. Determination of weight loss and lignin content of wood chips

After incubation, the mycelium attached to wood chips was removed. The wood chips were oven-dried at 50°C and weighed to calculate weight loss. The dried wood chips were milled to 40-mesh powder for the lignin content analysis. The lignin content was determined using the Klason lignin method (TAPPI Method T249 cm-85, 1999). Approximately 200 mg of milled wood was weighed accurately in a reaction flask. Then, 3 mL of 72% sulfuric acid (w/w) were added into the reaction flask and mixed well with a stirring rod

every 10 min for 2 h. The mixture was transferred into a septa-sealed bottle with 112 mL of deionized water to dilute an acid concentration of 3%. The mixture was autoclaved at 121°C for 1 h and placed on the bench overnight in order to allow the insoluble material to settle down as brown precipitate. The cooled reaction solution was filtered through preweighed sintered glass crucibles. Then, 10 mL of filtered solution was transferred into a sealed vial to analyse acid solution lignin content. The remaining solids in the bottle were recovered with 200 mL of warm deionized water into a sintered glass crucible. The glass crucible was placed overnight on oven-dryer at 105°C. Then, acid-insoluble lignin was determined from the wight of the dried crucible and calculated using the following equation.

Calculation of acid insoluble lignin content:

% acid insoluble lignin = (Wc / W) *100

Wc is oven-dried weight of acid insoluble lignin (g) and W is oven-dried weight of sample (g).

Acid-soluble lignin was quantified by UV-Vis spectroscopy at 205 nm. The acid solution obtained from the filterate was measured using a 3% sulfuric acid as a blank. If the absorbance was greater than 0.7, the filterate solution was further diluted to obtain an absorbance within 0.2

- 0.7. Then, acid soluble lignin content was calculated according to the following equation.

Calculation of acid soluble lignin content:

C (g mL^{-1}) = A / 110

% acid soluble lignin = (C * V) / (1000 * W) * 100

A is absorbance, V is total volume of filtrate, and W is oven-dried weight of sample (g).

Comparative statistical analysis was performed with the analysis of variance (ANOVA) test.

4.2.3. Enzymatic saccharification

To compare the fermentable sugar yields from the biomasses pretreated with the transformant and wild type strain, cellulase from *Trichoderma reesei* ATCC 26921 (Sigma, USA) and cellobiase from *Aspergillus niger* (Sigma, USA) were employed for the saccharification. Biologically pretreated woody biomass was ground to pass through a 0.40 mm screen. Then, 0.1 g of ground wood product was transferred to a 100 mL Erlenmeyer flask, and 20 mL of 50 mM sodium acetate buffer (pH 5.0) was added. Next, appropriate amounts of cellulase (80

EGU/g) and cellobiase (72 IU/g) were added. The flask was placed in a shaking incubator at 50°C and 150 rpm and incubated for 24 h. After hydrolysis, 2 mL of the incubation supernatants was centrifuged and filtered through a 0.45-μm filter. Monosaccharides were then determined using high-performance anionic exchange chromatography (HPAEC) (DionexBio-LC50 system; Sunnyvalle, USA). The Carbo Pac PA10 Column (4×250 mm) and ED 50 pulsed amperometric detector (PAD) were used. Chromatography was performed using 3 mM NaOH as the mobile phase at 0.8 mL/min for 45 min. Arabinose, galactose, glucose, xylose, and mannose were used as standard monosaccharides. Total sugar yield of *P. densiflora* and *L. tulipifera* was estimated as the sum of monosaccharide contents of unpretreated oven-dried wood chips (Table 4-1). The maximum yield of monosaccharides was analyzed using the filtrate of Klason lignin measurement after acid hydrolysis of unpretreated wood chips according to NREL Laboratory Analytical Procedures (Sluiter et. al., 2008). Two milliliters of the filtrate obtained from acid digestion with sulfuric acid was filtered with a 0.45-μm filter, and analyzed for the total sugar concentration using high-performance anionic exchange chromatography (HPAEC).

Table 4-1. Monosaccharide concentrations of wood chip

Species	Monosaccharide concentrations (%)[a]					
	Arabinose	Galactose	Glucose	Xylose	Mannose	Total
P. densiflora	-	4.9	35.9	5.4	10.7	57.1
	-	±0.1	±1.4	±0.1	±0.32	±1.6
L. tulipifera	0.5	0.6	39.8	15.9	3.5	60.3
	±0.01	±0.01	±0.5	±0.3	±0.1	±0.8

[a] Values are expressed as the percent of monosaccharide content based on the oven-dried weight of wood.

4.3. Results and Discussion

4.3.1. Enhanced lignin degradation by the transformants

Weight loss of the woody biomass as a result of the pretreatment with the wild type (WT) and the transformants is shown in Figure 4-1. *P. densiflora* (red pine) and *L. tulipifera* (tulip tree) were used as softwood and hardwood, respectively, for pretreatment. When *P. densiflora* wood chips were treated with the wild type strain, the transformant T21 and the transformant T26, the weight losses after 45 days were 2.8%, 4.3% and 5.8%, respectively (Figure 4-1a). In the case of *L. tulipifera* wood chips, the weight losses after biodegradation for 45 days were 30% by WT, 37% by T21 and 39% by T26. The hard wood exhibited greater weight loss than the softwood after the pretreatment with all strains. Although the pretreatment period and the wood species were different, this phenomenon is consistent with previous reports using *Pinus strobus* as softwood and *L. tulipifera* as hardwood (Hwang et al., 2008), and with *Cunninghamia lanceolata* as softwood and *Salix babylonica* as hardwood (Yu et al., 2009). In this study, the weight losses of the two woods by the transformants were higher than those by the wild type strain. Statistical analysis revealed that the degrees of weight loss after 15 days were not much different

among the strains tested. However, the degrees of lignin degradation by the pretreatment with the transformants were much enhanced (Figure 4-2). The highest lignin losses of *P. densiflora* by the transformants were 10% for 15 days and 14% for 45 days, which were 2 and 1.4 fold higher than those by the wild type. In *L. tulipifera,* the highest lignin losses were 16% for 15 days and 42% for 45 days, which were 3.2 and 1.4 fold higher than those for the wild type. In comparison with the previous research using *P. densiflora* (Lee et al., 2007a), lignin loss (14%) by transformants after 45 days were greater than the previously reported, specifically, 11.6% lignin loss which obtained after 56 days pretreatment by *P. brumalis.* Lee et al. (2007a) pretreated *P. densiflora* with three white-rot fungi, *C. lacerata, S. hirsutum* and *P. brumalis.* After 8 weeks of pretreatment, the least lignin loss was 11.6% with *P. brumalis*, while the greatest lignin loss was 14.5% with *S. hirsutum*. This result was due to the fact that *P. brumalis* ligninase activities were less than those of *S. hirsutum* during *P. densiflora* degradation. During *Acacia mangium* wood chip biodegradation with *Phellinus* sp., *Daedalea* sp., *T. versicolor* and *Pycnoporus coccineus*, laccase activities were much greater than those of manganese peroxidase and lignin peroxidase at 10 and 20 days (Liew et al., 2011). However, after

30 days, manganese peroxidase activities peaked and laccase activities were very low (Liew et al., 2011). Therefore, laccase produced during the initial degradation stage might be primarily responsible for lignin depolymerization. In this study, the transformants enhanced laccase activity by 3-4 fold compared to that of the wild type (Figure 2-10). Thus, the degradation ability of lignin from the transformants was improved in both soft and hard wood (Figure 4-1b) because transformants enhanced the activity of laccase. Hyphae of white-rot fungi penetrate adjacent cells through pore holes or pits. Fungal degradation of the cell wall components proceeds from the cell lumen toward the compound middle lamella, with gradual thinning of the cell wall. Softwood cell wall is composed of tracheids, parenchyma and epithelial cells, whereas hardwood consists of fibers, vessels, trachieds, parenchyma and epithelial cells (Fengel and Wegner, 1984). The pores that the hyphae are able to penetrate are larger than intersectional hardwood fibers. Therefore, the weight loss for the tulip tree hardwood was greater than that for the red pine softwood. The lignin is uniformly distributed across the middle lamella and the secondary wall of the wood. Degradation of the secondary wall proceeds more rapidly, whereas, at the compound middle lamella, fungal degradation is

retarded to a certain extent depending on the wood species. In the case of softwood, the lignin concentration in the middle lamella is higher (0.6-1.0 g/g) than that for hardwood (0.43-0.6 g/g) (Higuchi, 1985). Thus, the lignin loss rate of the soft wood chips by the transformants was lower than those of hard wood but the increasing rate of lignin loss by the transformants was higher in the soft than hard wood chips. The efficiency of loss of lignin was similar to the weight loss fraction of the wood chips.

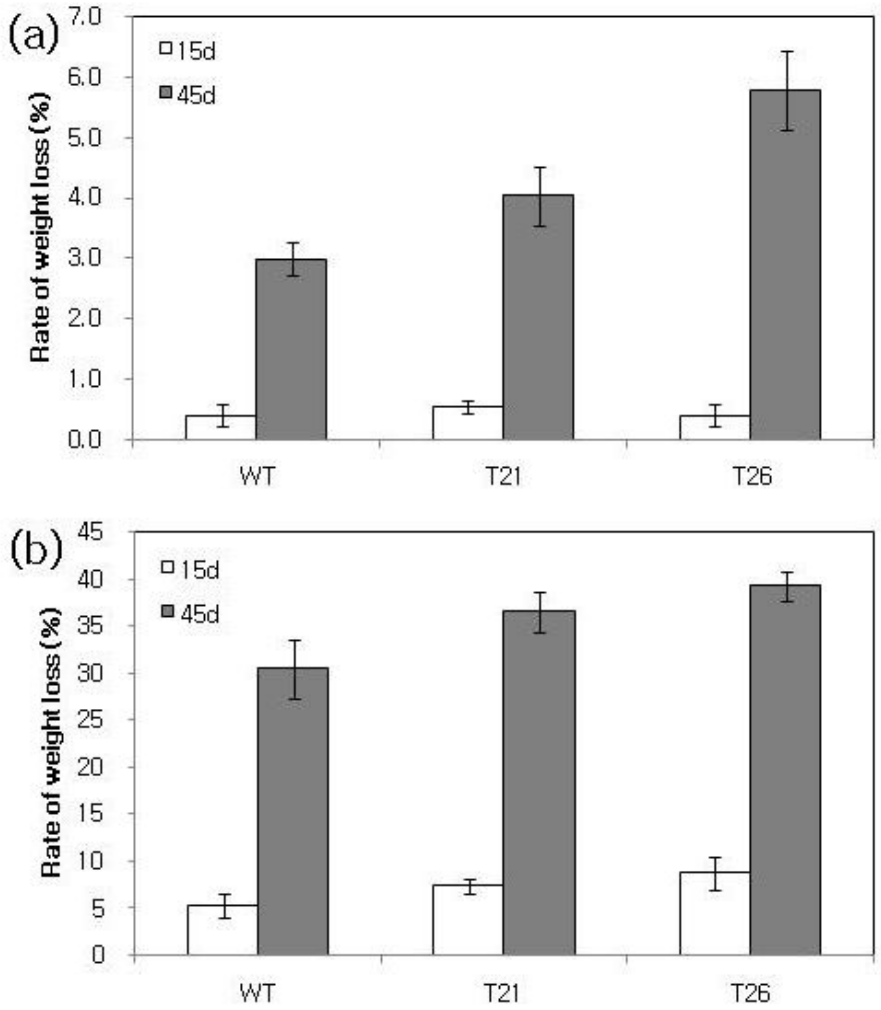

Figure 4-1. Weight loss of *P. densiflora* (a) and *L. tulipifera* (b) after the pretreatment with the wild type strain and the transformants for 15 and 45 days. The values represent the mean ± SE of 3 measurements with 3 replicates.

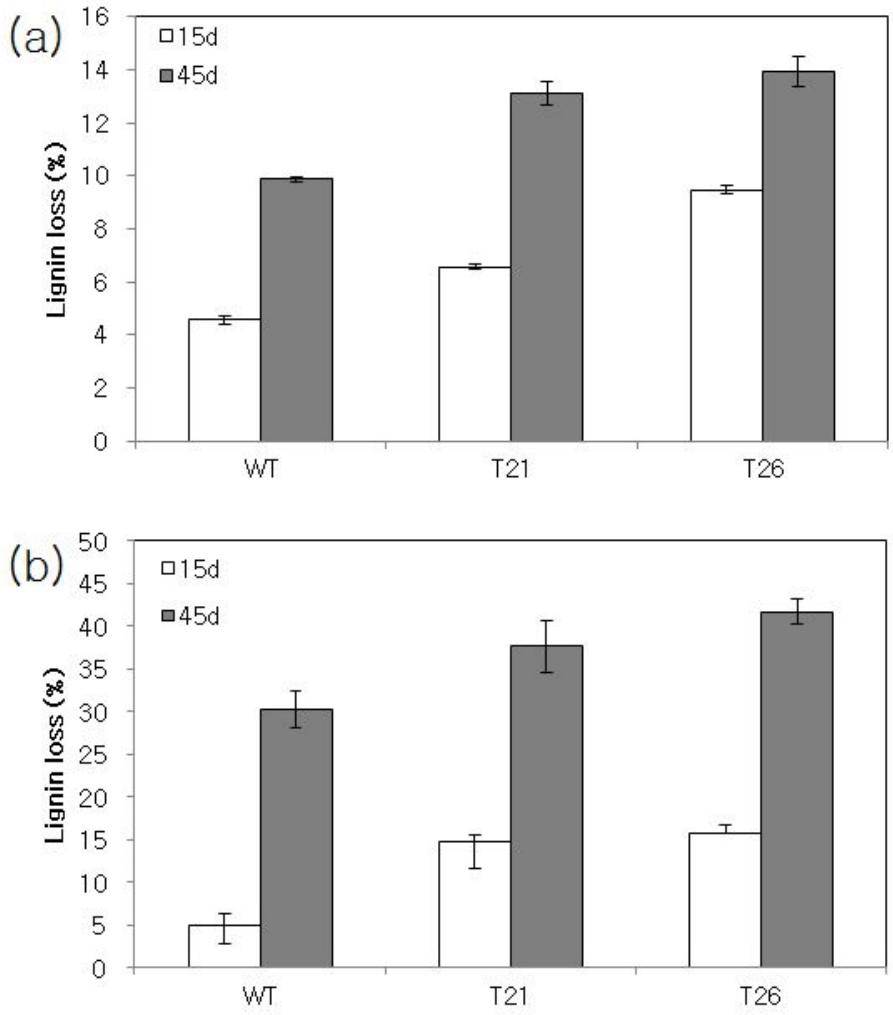

Figure 4-2. Lignin loss of *P. densiflora* (a) and *L. tulipifera* (b) after the pretreatment with the wild type strain and the transformants for 15 and 45 days. The values represent the mean ± SE of 3 measurements with 3 replicates.

4.3.2. Sugar yield of pretreated wood chips

The compositions of monosaccharides after the enzymatic saccharification of unpretreated and pretreated wood chips are presented in Table 4-2. According to the result, the concentrations of glucose were highest in monosaccharides and significantly increased with the pretreatment type. Xylose in *L. tulipifera* and mannose in *P. densiflora*, which are derived from glucomannan and arabinoglucuronoxylan as the main components of hemicellulose were the second highest after glucose, respectively. Arabionose was not detected in all samples. The enzymatic saccharification of pretreated wood with fungi gave rise to different yields of total sugars, including glucose, arabinose, xylose, galactose and mannose (Figure 4-3). The pretreatment with the wild type for 15 days resulted in an increase in the saccharification yield by a 3.8 fold for *P. densiflora* and a 4.2 fold for *L. tulipifera* as compared to the untreated control. When the *P. densiflora*-pretreated wood chips were treated with commercial cellulase, the highest amount of sugars obtained was 345 mg L^{-1} by T26, which was a 1.7 fold higher than that for the wild type (207 mg L^{-1}). For *L. tulipifera*, the highest sugar concentration was 464 mg L^{-1} by T26, which is a 1.9 fold increase compared to that for the wild type.

Lee et al. (2007a) showed that the pretreatment with *S. hirsutum*, which exhibited the highest ligninase activity, increased the available pore size to over 120 nm for *P. densiflora*, which in turn promoted adsorption of hydrolysis enzymes and efficient saccharification. The degradation of lignin by the ligninolytic enzyme from fungi was responsible for the increased available pore size. Thus, a saccharification yield in the transformant-pretreated wood may have been promoted by enhanced ligninolytic activity of laccase in the transformant.

Table 4-2. Compositions of monosaccharides after the enzymatic saccahrification of unpretreated and pretreated wood chip

Species	Pretreatment		Compositions of monosaccharides (mg L^{-1})				
			Arabinose	Galactose	Glucose	Xylose	Mannose
P. densiflora	Control		-	5.5 ± 2.4	24.5 ± 9.3	4.3 ± 2.7	9.1 ± 4.4
	WT	15 d	-	14.6 ± 5.7	104.5 ± 8.8	15.9 ± 2.4	40.5 ± 6.9
		45 d	-	19.2 ± 2.7	126.1 ± 34.9	20.1 ± 2.7	42.0 ± 7.6
	T26	15 d	-	21.5 ± 1.5	229.2 ± 23.6	24.9 ± 3.2	47.7 ± 5.5
		45 d	-	24.0 ± 3.1	246.9 ± 16.7	24.4 ± 2.9	50.2 ± 11.2
L. tulipifera	Control		-	3.4 ± 0.8	23.1 ± 8.6	11.5 ± 3.5	8.0 ± 4.4
	WT	15 d	-	8.5 ± 1.8	124.6 ± 7.8	41.0 ± 2.6	18.3 ± 3.6
		45 d	-	9.9 ± 2.2	164.3 ± 28.6	48.7 ± 9.9	20.8 ± 6.7
	T26	15 d	-	9.9 ± 1.6	213.7 ± 10.8	71.4 ± 3.3	23.5 ± 4.3
		45 d	-	12.3 ± 1.2	324.5 ± 25.7	89.2 ± 7.1	38.3 ± 4.3

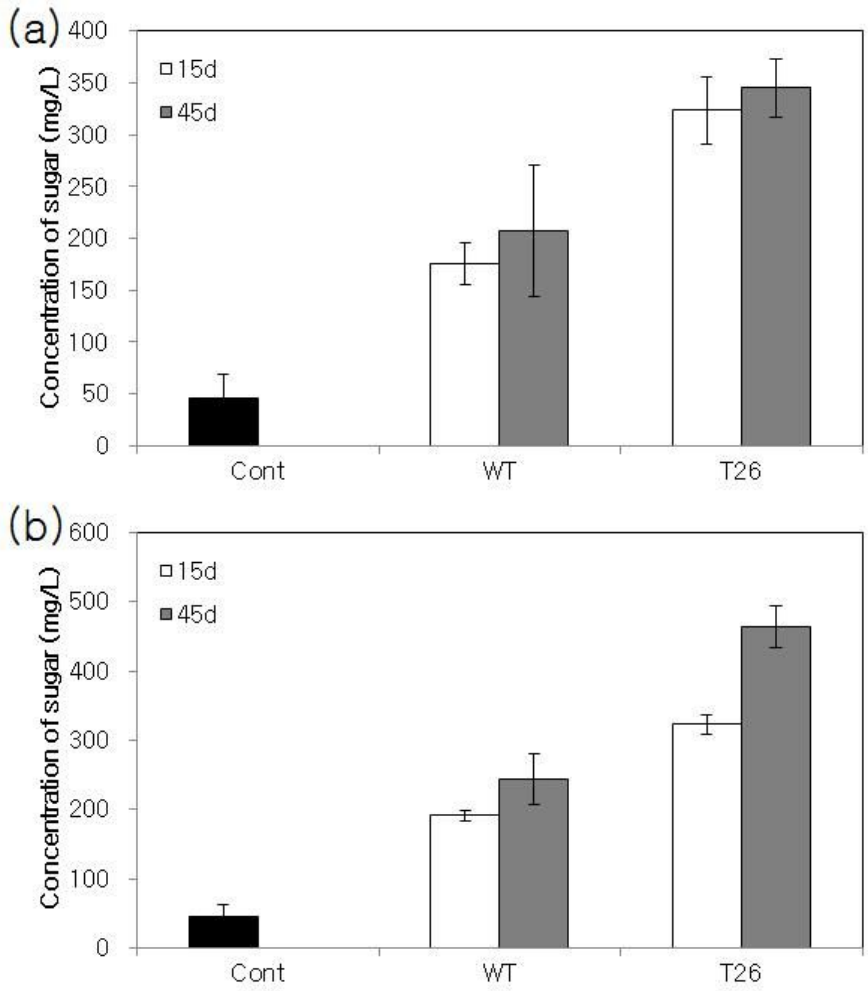

Figure 4-3. Amount of total sugar after the enzymatic saccharification of *P. densiflora* (a) and *L. tulipifera* (b) pretreated with the wild type strain and the transformant for 15 and 45 days. The control had not been pretreated with the fungi. The values represent the mean ± SE of 3 measurements with 3 replicates.

Considering the weight loss of the wood chip during pretreatment, the sugar yield (%) was calculated based on the dried wood weight used for pretreatment (Table 4-3). A sugar yield of T26 transformant for the pretreatment with *P. densiflora* for 15 days were similar to that obtained at 45 days although a sugar concentration of the 45 days pretreatment was higher than that of 15 days as shown in Figure 4-3a. In the case of *L. tulipifera,* sugar yields at 15 days pretreatment were higher than those at 45 days. This is due to the weight loss of wood chips, which also includes the loss of cellulose converted to sugars. The sugar concentrations of *L. tulipifera* pretreated with the T26 transformant for 15 and 45 days were 318 mg L^{-1} and 464 mg L^{-1}, respectively, or these were calculated as 295 mg and 283 mg based on 1 g of initial dried wood chip, which were corresponding to 29.5% and 28.3% in sugar yield (Table 4-3). The pretreatment for 45 days revealed much higher biomass weight loss (39%) than that for 15 days (7.4%), which caused a decreased total sugar yield based on the oven-dried original wood of *L. tulipifera*. Thus, for high recovery of sugars from lignocellulosic biomass, the pretreatment used for fungi should have an ability to degrade lignin with minimal effects on cellulose loss. In previous study, ligninase activities of *S. hirsutum* were the highest

while the activities of the cellulolytic enzymes were similar to those of other fungi (Lee et al., 2007a). *P. densiflora* pretreated with *S. hirsutum* showed the highest sugar yield at 21.01% from dry weight of biomass, which was a 1.4 fold higher than that (14.91%) of *P. brumalis*. However, the laccase-overexpressed transformant T26 showed 32.2% sugar yield at 15 days, which was a 1.9 fold higher than that of the wild type *P. brumalis* (Table 4-3), and 1.5 fold higher than that of *S. hirsutum*. Thus, enhanced ligninolytic activity of the transformant contributed to the increase of sugar yields for the conversion of lignocellulosic biomass.

The total monosaccharide contents of *P. densiflora* and *L. tulipifera* were 57.1% and 60.3% after acid hydrolysis (Table 4-1), which were regarded as maximum yields of total sugar in this study. Thus, the highest sugar recovery of *P. densiflora* and *L. tulipifera* pretreated with the transformant T26 are 56.9% and 48.9% of the maximum yield, respectively (Table 4-3). Koo et al. (2012) pretreated *L. tulipifera* using 1% sulfuric acid and 50% ethanol at 120℃, and obtained the highest sugar yield of 55% based on the carbohydrate composition of *L. tulipifera* after the enzymatic saccharification. The sugar yield (55%)

by the chemical pretreatment of sulfuric acid and ethanol was higher than the sugar yield (48.9%) by the biological pretreatment of the transformant T26. However, the sugar yield by the transformant T26 was reasonable when considering the lower cost and simple reaction conditions.

Table 4-3. Total sugar yields of unpretreated and pretreated woody biomasses by commercial enzymes

Species	Maximum sugar yield[a] (%)	Sugar yield after enzyme saccharification[b] (%)				
		Control	Biological pretreated			
			15 d		45 d	
			WT	T26	WT	T26
P. densiflora	57.1 ± 1.6 (100)[c]	4.3 ± 2.2 (7.5)	17.4 ± 2.0 (30.5)	32.2 ± 3.2 (56.4)	20.2 ± 6.1 (35.4)	32.5 ± 2.7 (56.9)
L. tulipifera	60.3 ± 0.8 (100)	4.6 ± 1.6 (7.6)	18.2 ± 0.7 (30.2)	29.5 ± 1.3 (48.9)	17.0 ± 2.6 (28.2)	28.2 ± 1.9 (46.8)

[a] Values are expressed as the percent of maximum sugar yield based on the oven-dried weight of wood.

[b] Values are expressed as the percent of sugar yield after the enzymatic saccharification based on the oven-dried weight of wood

[c] Values in parentheses are expressed as the percent of sugar yield based on maximum sugar yield.

Some transformants with the ligninase gene were used for the degradation of endocrine disrupting chemicals (EDCs), which mimic and interfere with the mechanisms of action of endogenous gonadal steroid hormones (Colborn et al., 1996; Yeo et al., 2008; Kum et al., 2011). The laccase activity of the *Irpex lacteus* transformant with the *P. tremellosa* laccase gene was 6 fold greater than that of the wild type strain and exhibited enhanced degradation activity in the case of EDCs (Kum et al., 2011). Genetic transformation with genes for laccase and MnP in *P. tremellosa* concurrently augmented laccase and MnP activity and rapidly degraded 2 EDCs, that is, bisphenol A and nonylphenol (Kum et al., 2011). To utilize white-rot fungus transformants for the pretreatment of lignocelluloses for biofuel production is more complicated than using them for the degradation of EDCs. The reason is that white-rot fungi consume cellulose as a carbon source along with lignin, resulting in low cellulose recovery from biomass. *T. versicolor* transformant MrP1 was able to overexpress the manganese-repressed peroxidase gene, exhibiting higher MnP activity and biomass weight loss than the wild type, but the glucose yield was lower than that of the wild type after pretreatment (Hwang et al., 2008). To develop cost-effective microbial pretreatment, the microbe's ability to delignify

lignocellulosic biomass needs to be improved but weight loss of biomass should be minimized during pretreatment. Optimal growth conditions might be also important for achieving high lignin loss and low cellulose loss in parallel.

4.4. Conclusions

Genetic transformation of *P. brumalis* with the laccase gene generated new strains with increasing ligninolytic activity. These transformants exhibited enhanced lignin-degradation ability during pretreatment of wood chips and incresed sugar yields from the enzymatic saccharification of the pretreated wood chips compared with the wild type strains. Therefore, the overexpression of the laccase gene in *P. brumalis* via genetic transformation is a useful tool for the improvement of the biological conversion of lignocellulosic biomass.

In the future, analysis of the biological pretreatment by the MnP transformants should be preformed. In addition, minimization of biomass loss during pretreatment by white-rot fungi might be developed for the cost-effective bioconversion of linocellulosic biomass.

OVERALL CONCLUSIONS

White-rot fungi produce lignin degrading enzymes which can efficeiently mineralise lignin of lignocellulosic biomass into CO_2 and H_2O. Therefore, applications of the fungi's capability can offer environmentally friendly processes to replace physical or chemical pretreatment for lignocellulosic biomass. In this study, genetic transformation of a white-rot fungus, *P. brumalis* has been performed to enhance its ligninolitic activity by introducing the genes coding lignin degrading enzymes isolated from *P. brumalis* and then, the transformants have been applied to the biological pretreatement of lignocellulosic biomass.

In Chpater II, two laccase cDNAs (*pblac1* and *pblac2*) were isolated from *P. brumalis*. The cloned cDNAs of laccase 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. An RT-PCR analysis revealed that the transcript levels of the two laccases increased when *P. brumalis* was exposed to DBP. The overexpression of the *pblac1* gene under the control of the *gpd* promoter was derived by the genetic transformation in *P. brumalis*. The specific activity of laccase in the

transformants was about 3 folds higher than that of the wild type. The transformants exhibited more effective decolorization of the dye Remazol Brilliant Blue R than the wild type.

In Chapter III, the cDNAs of six manganese peroxidases (*pbmnp1-6*) were isolated from *P. brumalis*. MnP proteins share similar properties with each other in terms of size (approximately 360-365 amino acids) and primary structure, showing 62-96% amino acid sequence identity. MnP activity increased by the treatment with dibutyl phthalate (DBP) and wood chips. The expression of *pbmnp5* was induced by DBP only, while that of *pbmnp6* was induced by wood chips only. Expression of *pbmnp4* was strongly induced by both treatments. The *pbmnp4* gene was overexpressed in *P. brumalis* under the control of the *gpd* promoter. Overexpression of *pbmnp4* effectively enhanced MnP activity; the transformant that had the highest MnP activity also demonstrated the most effective decolorization of Remazol Brilliant Blue R dye.

In Chapter IV, when the transformants that overexpressed the laccase gene, *pblac1*, were incubated with wood chips from a red pine (*P. densiflora*; softwood) and a tulip tree (*L. tulipifera*; hardwood) for 15 and 45 days, they exhibited higher lignin-degradation activity as well as higher wood-chip weight loss than the wild type. When the wood chips

treated with the transformant were enzymatically saccharified, the highest sugar yields were found to be 32.5% for the red pine wood and 29.5% for the tulip tree wood, based on the dried wood weights, which were 1.6 folds higher than those for the wild type. These results suggested that overexpression of the laccase gene in *P. brumalis* significantly contributed to pretreatment of lignocellulose for increasing sugar yields.

In this study, characterization of laccase and MnP cDNAs has allowed to the construction of the genetic transformants with enhanced ligninolytic activity in *P. brumalis*. The laccase transformants of *P. brumalis* have improved lignin degradation ability and sugar yields for the pretreatment of wood chips. Despite these positive results, a number of problems remain to be examined including the analysis of the biological pretreatment by the MnP transformants, the minimization of biomass loss during pretreatment to develop the cost-effective biological pretreatment with white-rot fungi, and the optimization of pretreatment conditions for achieving high lignin loss and sugar yield of lignocellulosic biomass.

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국문초록 (ABSTRACT IN KOREA)

본 연구에서는 백색부후균인 겨울우산버섯으로부터 리그닌분해에 관련된 유전자를 분리한 후 재조합을 통해 그 유전자를 과발현시킨 겨울우산버섯의 형질전환체를 제작하였고 이를 이용한 목질계 바이오매스의 전처리 효과를 조사하였다.

먼저 리그닌 분해효소 유전자를 확보하기 위해 국립산림과학원에서 보관하고 있는 겨울우산버섯(KFRI 20912) 균주로부터 RT-PCR과 RACE 방법으로 2개의 락케이스와 6개의 망간퍼옥시데이스의 cDNA를 분리 하였다. 겨울우산버섯의 균사내에서 유전자들의 RNA 발현을 조사한 결과, PDB배지에서 보다 SSC 배지에서 배양할 때 모든 유전자들의 발현이 높았고 리그닌분해효소 활성도 높았다.

리그닌과 유사한 난분해성 화학구조를 하고 있는 다이부틸 프탈레이트(DBP)를 처리하였을 때 모든 유전자의 발현이 증가되었고, 리그닌을 구성물질로 갖는 목재칩의 처리에 의해 망간퍼옥시데이스 유전자들의 발현이 증가되었다. 따라서 분리된 유전자들이 리그닌을 포함한 난분해성 물질의 분해에 관여하는 것으로 판단 할 수 있었고, 또한 락케이스 중에서는 *pblac1* 유전자와 망간퍼옥시데이스 중에서는 *pbmnp4* 유전자가 다른 유전자들에 비해 발현이 높았고 효소의 활성과 경향이 일치하였으므로 이들의 유전자 발현이 각각의 효소활성에 중요한 영향을 미친다는 것을 추정할 수 있었다.

락케이스와 망간퍼옥시데이스의 활성이 증가된 겨울우산버섯을 얻기 위해 *pblac1*과 *pbmnp4* 유전자를 이용하여 각각의 형질전환체를 제작하였다. 항상 유전자의

발현을 유도하는 glyceraldehyde-3-phosphate dehydrogenase (*gpd*) 프로모터의 조절에 의해 *pblac1*과 *pbmnp4*가 과발현하는 형질전환체는 야생형 균주보다 락케이스와 망간퍼옥시데이스의 효소활성이 크게 증가되었으며 Remazol Brilliant Blue R 염료의 탈색능도 야생형 균주보다 훨씬 높게 나타내었다. 따라서 리그닌분해효소 유전자의 과발현을 통해 형질전환체의 리그닌 분해능이 증가되었음을 간접적으로 알 수 있었다.

락케이스가 과발현되는 형질전환체로 소나무와 백합나무의 목재칩을 15일과 45일 동안 전처리한 결과 목재의 중량감소와 리그닌 분해율이 야생형 균주로 전처리한 것보다 높은 것을 확인하였다. 형질전환체로 전처리한 목재칩을 효소당화 시켰을때 당 수율은 소나무에서 건조무게의 32.5% 그리고

백합나무에서 건조무게의 29.5%로 야생형균주로 전처리한 것보다 약 1.6배 증가되었다. 이런 결과는 겨울우산버섯에서 형질전환에 의한 락케이스 과발현이 목질계 바이오매스의 전처리 효율과 당수율 증가에 기여했다는 것을 보여준다.

결론적으로 락케이스와 망간퍼옥시데이스 유전자의 분리 및 특성 구명은 백색부후균의 리그닌 분해와 관련된 분자메카니즘을 이해할 수 있는 시발점이 될 수 있으며, 형질전환 기술을 통해 리그닌분해효소 활성을 높임으로써 목질계 바이오매스의 생물학적 전처리에 백색부후균을 효과적으로 활용할 수 있게 될 것이다.

주요어 : 겨울우산버섯, 락케이스, 망간 퍼옥시데이스 (MnP), 목질계 바이오매스, 형질전환체, 전처리

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