RESEARCH LETTER

Increased expression of laccase by the addition of phthalates in *Phlebia tremellosa*

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Abstract

Phthalates, which are used as plasticizers in the plastics industry, are widely used and have been dispersed into the environment. A white-rot basidiomycete *Phlebia tremellosa*, which showed good growth in media containing various hormone-mimicking compounds, degraded benzylbutylphthalate and diethylphthalate by up to 30% and 80%, respectively, under liquid culture conditions for 9 days. A laccase cDNA from *P. tremellosa* was cloned by a rapid amplification of cDNA ends (RACE)-PCR technique, and found to encode 1832 nucleotides. Its deduced amino acid sequence showed 80.7% identity when compared with that of *Phlebia radiata*, and 64.8% identity when compared with that of *Trametes versicolor*. When this fungus was grown under suitable conditions for degrading phthalic esters, the laccase activity and its transcript level were both highly increased.

Introduction

As the consumption of disposable products such as plastics increases, the environment is becoming more and more contaminated by steroid hormone-mimicking compounds (HMCs) and endocrine-disrupting compounds (EDCs), which interfere with normal hormonal activities. Many diverse HMCs are found in groundwater, lakes and soils, and because it is very difficult to degrade these chemicals they can easily become concentrated in the human body. Phthalic acid esters are widely used in the cosmetics and plastics industries. Bisphenol A has received much attention from many scientists owing to its large production and has been used for dental sealants, epoxy resins, polycarbonates and various plastics (Staples *et al*., 1998). Even at very low concentrations, these HMCs interfere with animal reproductive systems (Schonfelder *et al*., 2002). Phthalates have been reported to acquire unequivocal estrogenic activities as a result of light exposure (Okamoto *et al*., 2006). As insecticides can also be a source of HMCs, there is a high risk that the air and groundwater will be polluted with HMCs. It is therefore very important to degrade these HMCs that disrupt human hormonal activity. There are reports of the degradation of HMCs by UV treatment (Chen *et al*., 2006), and by biological methods using bacteria (Soares *et al*., 2003; Li *et al*., 2006) and microalgae (Hirooka *et al*., 2003). The biological degradation of toxic compounds has a positive effect, in spite of its slow removal rate, because there is no need to use other chemicals to remove contaminating HMCs.

Lignin-degrading white-rot basidiomycetes have enzyme systems for the degradation of lignocelluloses, and these enzymes have also been used for the degradation of various recalcitrant compounds such as polyaromatic hydrocarbon compounds (Han *et al*., 2004), halogenated hydrocarbon compounds (Reddy & Gold, 2000; Mori *et al*., 2003), and many recalcitrant compounds (for a review, see Baldrian, 2006). Several enzymes are involved in these degradations, including laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and glucose oxidase, which supplies H_2O_2 for the peroxidase reactions. Laccase (benzenediole: oxygen oxidoreductase, EC1.10.3.2) is widely distributed in higher fungi, and it has been reported that this enzyme shows very diverse biological functions. It is related to the conidiophore pigmentation, and therefore its expression is regulated by the developmental stage in *Aspergillus nidulans* (Clutterbuck,

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1990; Scherer & Fischer, 1998). In the case of *Cryptococcus neoformans*, which is a pathogenic fungus to humans, the survival of the fungal cell by means of the laccase reaction inside a human macrophage is linked to the fungal pathogenicity (Panepinto & Williamson, 2006).

There have been many attempts to analyse the roles of laccases in the degradation of lignin and other recalcitrant compounds by white-rot fungi. When *Trametes versicolor* was grown with 2,4,6-trinitrotoluene (TNT) and its catabolic intermediates, the fungal culture showed increased laccase activity as well as highly induced expression (Cheong *et al.*, 2006). Laccase was greatly induced by xenobiotic 2,5-xylidine in *T. versicolor* (Kollmann *et al.*, 2005). We isolated a white-rot basidiomycete, *Phlebia tremellosa*, from a forest near Seoul, and found it to show good growth on potato dextrose agar (PDA) plate with several HMCs. When this fungus was grown in a liquid medium containing HMCs, laccase and lignin peroxidase showed increased activities. We analysed the degradations of phthalic esters in a liquid culture of *P. tremellosa* by high-performance liquid chromatography (HPLC). Here we also report the cloning of laccase cDNA and its increased expression in liquid media containing several HMCs.

Materials and methods

Fungal strain and growth conditions

Phlebia tremellosa monokaryon (Pt05-2) was isolated from the dikaryotic mycelium through the protoplast generation and regeneration method (Kim & Choi, 1995). The fungus was grown at 30 °C in PDA medium, and a minimal medium was used when needed. The minimal medium used in the degradation experiment had the following composition: glucose 10 g, ammonium tartrate 1 g, KH_2PO_4 0.5 g, $CaCl_2$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, mineral solution 5 mL, and thiamine HCl solution 5 mL in d-H_2O 1 L. Mineral solution consisted of NaCl 1 g, $MnSO_4 \cdot 5H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.1 g, $CoCl_2 \cdot 6H_2O$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, $CuSO_4 \cdot 5H_2O$ 0.1 g, $AlK(SO_4)_2 \cdot 12H_2O$ 10 mg, H_3BO_3 10 mg, $Na_2MoO_4 \cdot 2H_2O$ 10 mg, nitrilotriacetate 1.5 g in d-H_2O 1 L. Thiamine HCl was dissolved 100 mg in d-H_2O 1 L. Pt05-2 was grown on PDA plate, and potato dextrose broth (PDB) or minimal liquid medium were inoculated with 20 pieces of mycelia cut with a #1 cork borer (diameter 4 mm). Benzylbutylphthalate (BBP, 98%), bisphenol A (BPA, 99+%) and diethylphthalate (DEP, 99.5%) were purchased from Aldrich.

Determination of the removal of HMCs from the fungal culture

A liquid culture of Pt05-2 was grown for 5 days by inoculating 10 pieces of mycelial block cut with a #1 cork borer, and the whole culture was ground with a Waring blender. The homogenized culture (10 mL) was transferred to 100 mL of the fresh minimal medium, and each HMC (100 μM mL^{-1}) was added at this point. The residual concentrations of the HMCs were determined as follows. The fungal culture including the cells and supernatant was extracted with n-hexane and then ethylacetate in a 50-mL centrifuge tube. The mixture was separated by centrifugation at 24 900 ***g*** for 20 min. The organic phase containing the residual HMCs was analysed by Waters HPLC (HP 1100 series, symmetric column). The flow rate was 0.6 mL min^{-1}, and the elution solvent was a mixture of acetonitrile and water (80 : 20). The retention time of each HMC was determined by monitoring the effluent, and confirmation of each HMC was made by comparison of the retention time of the analytical-grade HMCs respectively.

Cloning of a laccase cDNA gene

Fungal laccases generally have four conserved copper-binding regions, and the base sequences of these regions have been used as the PCR primers (D'Souza *et al.*, 1996; Cheong *et al.*, 2006). Two degenerated primers, primer 1 as a forward primer 5′-CAYTGGCAYGGNTTYTTYCA-3′ and primer 2 as a reverse primer 5′-TGNCCGTGMARRTGSAANGG-3′, which represent the copper-binding domains I and III, respectively, were used. Total RNAs were isolated according to a previous report (Cheong *et al.*, 2006), except using a 2-day-old shake culture in PDB medium. The first strand of cDNA was synthesized from 1 μg of total RNA using PowerScript reverse transcriptase (Promega) by following the manual for CapFishing cDNA isolation kit (Seegene, Korea), and PCR was performed using Taq polymerase with the two primers. The 5′-region of the cDNA was synthesized using the 5′-RACE-PCR (rapid amplification of cDNA ends-PCR) primer and a specific reverse primer (5′-GATGGTGAAATT GAACCACGAGTCACAGGA-3′), and the 3′-region was amplified using the 3′-RACE-PCR primer and a specific forward primer (5′-TGATCCTTATGCGGATTTGTACGAC GT TG-3′). Full-length cDNA was generated by two-step PCR following the manufacturer's protocol.

Determination of laccase gene expression under degradation conditions of HMCs

Phlebia tremellosa was grown in the minimal liquid medium for 5 days, and transferred to the same liquid medium with the fungal pellets homogenized by a Waring blender. BBP (1 mM), BPA (0.2 mM) and DEP (1.8 mM) were added with the inoculums, and the cultures were grown with shaking at 30 °C. Culture supernatants were removed for the enzyme assay, and fungal pellets were collected in order to isolate total RNAs for the Northern analysis on day 7. Laccase assay in the culture supernatants was performed as in a previous

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report using *o*-tolidine as the chromogenic substrate (Ko *et al.*, 2001). Total RNAs from each culture (10 μg) were separated on the denaturing RNA gel, and transferred to a nylon membrane. Northern analysis for the gene expression was performed with the DIG-DNA-labelled probe generated by the Klenow reaction using the cloned cDNA as template.

Results and discussion

White-rot fung that have enzyme systems for the degradation of lignin have a high potential in the treatment of toxic recalcitrant compounds. For *T. versicolor*, laccase and MnP have been shown to be involved in dye decolouration (Champagne & Ramsay, 2005), and bisphenol A and nonylphenol were degraded by MnP and laccase with a mediator from white-rot fungi (Tsutsumi *et al.*, 2001). The utilization of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfoninc acid) as a mediator for laccase significantly increased the enzymatic degradation of nonylphenol, bisphenol A and triclosan by *Coriolopsis polyzona* (Cabana *et al.*, 2007). *Phlebia tremellosa* was selected from many white-rot fungi isolated in Korea because this fungus showed relatively good growth (ca. 80% growth at the indicated concentrations of HMCs) on agar medium containing phthalates and bisphenol A when compared with *Stereum ostrea*, *Polyporus brumalis* and *Schizophyllum commune* (60–70% growth). Because the inhibition effect of each HMC on the fungal growth was different, we used the concentration of each HMC that allowed the same amount of mycelial growth. The fungus showed about 85% growth in the liquid minimal medium containing BBP (1 mM), BPA (0.2 mM) or DEP (1.8 mM), respectively, when the growth of each culture was determined by measuring changes of the cell dry weight (data not shown). BBP was transformed quite rapidly, showing more than 50% removal after incubation for 2 days, and above 80% was transformed on day 9 by this fungus. DEP was transformed more slowly than BBP, reaching 30% removal on day 9 (Fig. 1). However, there was almost no degradation of BPA by this fungus (data not shown). It is necessary to determine the residual estrogenic activity of the catabolic intermediates during the transformation of HMCs by the fungal enzyme systems.

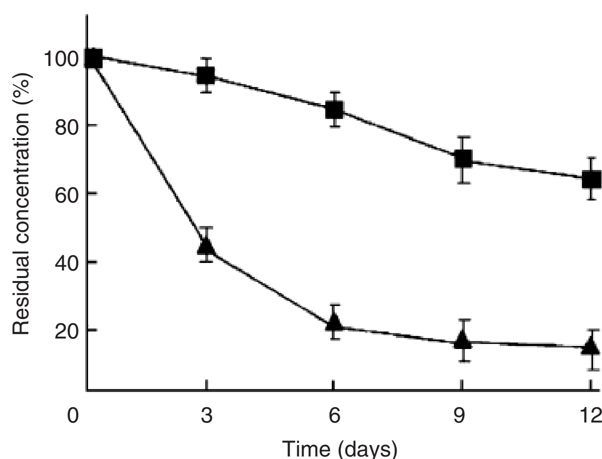

Fig. 1. Transformation (removal) of phthalic esters (BBP and DEP: 100 μM each) in the minimal medium of *Phlebia tremellosa*. Triangles, BBP; squares, DEP.

```
GGGGGATAGGGGGGGAGGAAGCGGAGTTCCCCGCTGACAAGGGGTTGCCAGAGGGCCTCT    60
      M  F  S  F  A  R  S  T  A  L  V  V  A  G  L  F  V  G
TCACCAATGTTTTCCTTCGCTCGTTCTACTGCGCTCGTCGTCGCAGGCCTTTTCGTTGGT   120
A  I  A  A  I  G  P  V  T  D  F  H  I  V  N  A  A  I  A  P
GCCATCGCCGCCATCGGCCCTGTGACTGACTTTCATATCGTCAATGCAGCCATCGCCCCC   180
D  G  I  S  R  Q  A  V  L  A  E  G  T  F  P  G  P  L  I  T
GACGGTATCTCTCGTCAGGCCGTGCTTGCTGAGGGCACATTCCCTGGTCCTCTCATTACT   240
G  N  K  G  D  N  F  Q  I  N  V  I  D  E  L  T  N  A  T  M
GGCAACAAGGGCGACAACTTCCAAATCAATGTTATCGACGAGCTAACCAATGCTACCATG   300
L  K  S  T  T  I  H  W  H  G  F  F  Q  H  G  T  N  W  A  D
CTCAAGTCGACGACGATCCACTGGCATGGATTCTTCCAGCACGGAACGAACTGGGCTGAT   360
G  P  A  F  V  N  Q  C  P  I  A  T  G  N  S  F  L  Y  N  F
GGTCCGGCCTTCGTCAATCAATGTCCTATTGCTACCGGGAATTCTTTCTTGTACAACTTC   420
N  A  P  D  Q  A  G  T  F  W  Y  H  S  H  L  S  T  Q  Y  C
AACGCACCCGATCAAGCCGGTACATTCTGGTATCATAGTCATCTGTCCACTCAGTACTGT   480
D  G  L  R  G  P  F  V  V  Y  D  P  N  D  P  Y  A  D  L  Y
GACGGTTTGCGTGGTCCTTTTGTGGTCTACGATCCCAATGATCCTTATGCGGATTTGTAC   540
D  V  D  D  D  S  T  V  I  T  L  A  D  W  Y  H  T  A  A  R
GACGTTGATGACGATTCTACCGTCATCACGCTGGCCGACTGGTATCATACGGCTGCCCGC   600
L  G  G  P  F  P  N  A  D  T  T  L  I  N  G  L  G  R  C  G
CTCGGCGGTCCTTTCCCCAATGCCGACACTACTTTGATTAATGGCCTGGGCAGATGCGGT   660
E  P  G  A  P  V  S  D  L  A  V  I  N  V  E  A  G  K  R  Y
GAACCGGGCGCTCCTGTCTCTGATCTCGCTGTTATCAACGTCGAGGCTGGCAAGCGTTAT   720
R  M  R  L  V  S  I  S  C  D  S  W  F  N  F  T  I  D  G  H
CGAATGCGCTTGGTTTCGATCTCCTGTGACTCGTGGTTCAATTTCACCATCGATGGACAC   780
D  M  T  I  I  E  V  D  G  V  N  H  E  T  L  T  V  D  Q  I
GATATGACCATAATTGAAGTTGATGGCGTCAACCACGAGACGTTGACCGTAGACCAAATC   840
Q  I  F  A  A  Q  R  Y  S  F  I  L  E  A  N  Q  P  V  D  N
CAGATCTTCGCCGCTCAGCGTTACTCTTTCATCTTGGAAGCAAACCAGCCTGTCGATAAC   900
Y  W  I  R  A  N  P  G  T  G  I  T  Q  G  F  E  G  G  I  N
TACTGGATTCGCGCAAACCCGGGTACCGGCATCACTCAAGGGTTCGAGGGCGGCATCAAC   960
S  A  I  L  R  Y  A  G  A  D  E  V  E  P  T  T  A  L  V  A
TCGGCCATTCTGCGCTATGCTGGCGCTGACGAGGTTGAACCTACCACTGCGTTAGTTGCG  1020
S  T  S  P  L  A  E  G  D  L  H  P  L  D  N  P  A  A  S  G
AGCACCTCTCCGTTGGCCGAGGGAGATTTGCACCCTCTAGACAACCCTGCTGCCNCTGGC  1080
Q  P  F  P  G  G  V  D  Y  A  L  N  L  N  L  T  F  D  G  P
CAACCCTTCCCAGGCGGCGTCGATTACGCTTTGAATTTGAACTTGACATTCGATGGACCC  1140
T  L  K  F  L  I  N  G  V  S  F  V  P  P  T  V  P  V  L  L
ACCCTTAAGTTCCTGATCAATGGGGTGTCTTTCGTACCCCCGACAGTTCCGGTGCTCCTG  1200
Q  I  L  S  G  A  Q  T  A  Q  D  L  L  P  T  G  S  V  Y  S
CAGATTCTCAGTGGTGCGCAGACCGCACAAGATCTTCTTCCTACTGGGAGTGTCTACTCC  1260
L  P  S  N  A  T  I  E  L  S  L  P  A  G  P  A  G  G  P  H
TTGCCCTCGAACGCGACCATTGAATTGAGTCTCCCTGCTGGTCCTGCAGGCGGACCTCAT  1320
P  F  H  L  H  G  H  T  F  S  V  V  Q  G  A  G  Q  T  V  P
CCCTTCCACTTGCACGGCCACACGTTCAGTGTGGTTCAAGGTGCAGGACAAACGGTGCCG  1380
N  Y  V  N  P  V  R  R  D  V  V  S  I  G  A  V  P  D  N  V
AACTACGTGAATCCTGTCCGCCGAGATGTTGTCAGCATCGGAGCTGTTCCCGACAATGTC  1440
T  I  R  F  E  T  N  N  P  G  P  W  F  L  H  C  H  I  D  W
ACCATTCGTTTCGAGACCAACAATCCCGGCCCATGGTTCCTCCATTGCCACATCGACTGG  1500
H  L  D  A  G  F  A  V  V  F  A  E  D  I  P  D  I  A  S  V
CACTTGGATGCTGGCTTCGCCGTGGTATTCGCTGAAGACATCCCCGATATCGCTTCGGTC  1560
S  P  V  P  T  D  W  S  N  L  C  P  I  Y  D  A  L  D  P  S
AGCCCTGTCCCTACCGACTGGTCCAATCTGTGCCCAATCTACGACGCTCTCGATCCTTCT  1620
D  Q  *
GACCAGTAAGGATTGGTATTGATATGCCGTTGGTGGCTGTGAACGCGGCGATGGACAATG  1680
GACTATAAGTATTTGCAGTATACATATATCTTTCACATCACAAGGGGGAACAGATATACG  1740
GACTAGACTTTTGGAGTACTGTTGGCTCAGTAGTGTAATTGATGAGGCGTCAGTGAAGCC  1800
CAGTGGTACCGCAAAAAAAAAAAAAAAAAAAA    1832
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Fig. 2. Cloned cDNA sequence and its deduced amino acid sequence of *placc1* of *Phlebia tremellosa*. Untranslated 5′- and 3′-regions are written in italics, and the * represents the stop codon. Four possible copper-binding domains are underlined.

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1 PT Lac  -MFSFARSTALVVAGLFVGAIAAIGPVTDFHIVNAAIAPDGISRQAVLAEGTFPGPLITG
2 PR Lac  -MHTFLRSTALVVAGLSARALASIGPVTDFHIVNAAVSPDGFSRQAVLAEGVFPGPLIAG
3 TV Lac  MGRFSSLCALTAVIHSFGRVSAAIGPVTDLTISNADVSPDGFTRAAVLANGVFPGPLITG
                      *        * ******  * **   ***  * **** * ****** *

1 PT Lac  NKGDNFQINVIDELTNATMLKSTTIHWHGFFQHGTNWADGPAFVNQCPIATGNSFLYNFN
2 PR Lac  NKGDNFQINVIDELTNATMLKTTTIHWHGFFQHGTNWADGPAFINQCPIASGDSFLYNFQ
3 TV Lac  NKGDNFQINVVDNLSNETMLKSTSIHWHGFFQKGTNWADGAAFVNQCPIATGNSFLYDFT
          ********** * * * **** * ******** ******* ** ****** * **** *

1 PT Lac  APDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPYADLYDVDDDSTVITLADWYHTAARL
2 PR Lac  VPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPADPYLDQYDVDDDSTVITLADWYHTAARL
3 TV Lac  ATDQAGTFWYHSHLSTQYCDGLRGPMVVYDPSDPHADLYDVDDETTIITLSDWYHTAASL
            *********************** ***** **  * *****  * *** ******* *

1 PT Lac  GGPFPN-ADTTLINGLGRCGEPGAPVSDLAVINVEAGKRYRMRLVSISCDSWFNFTIDGH
2 PR Lac  GSPFPA-ADTTLINGLGRCGEAGCPVSDLAVISVTKGKRYRFRLVSISCDSFFTFSIDGH
3 TV Lac  GAAFPIGSDSTLINGLG--RFAGGDSTDLAVITVEQGKRYRMRLLSLSCDPNYVFSIDGH
          *  **   * *******     *    ***** *  ***** ** * ***    * ****

1 PT Lac  DMTIIEVDGVNHETLTVDQIQIFAAQRYSFILEANQPVDNYWIRANPGTGITQGFEGGIN
2 PR Lac  SLNVIEVDATNHQPLTVDELTIYAGQRYSFILTADQDVDNYWIRANPGIGITTGFAGGIN
3 TV Lac  NMTIIEADAVNHEPLTVDSIQIYAGQRYSFVLTADQDIDNYFIRALPSAGTTS-FDGGIN
              ** *  **  ****   * * ***** * * *  *** *** *  * *  * ****

1 PT Lac  SAILRYAGADEVEPTTALVASTSPLAEGDLHPLDNPAAXGQPFPGGVDYALNLNLTFDGP
2 PR Lac  SAILRYDGADVVEPTTTQATSPVVLSESNLAPLTNAAAPGLPEVGGVDLALNFNLTFDGP
3 TV Lac  SAILRYSGASEVDPTTTETTSVLPLDEANLVPLDSPAAPGDPNIGGVDYALNLDFNFDG-
          ****** **  * ***    *   * *  * **   ** * *  **** ***    ***

1 PT Lac  TLKFLINGVSFVPPTVPVLLQILSGAQTAQDLLPTGSVYSLPSNATIELSLPAGPAGGP-
2 PR Lac  SLKFQINGVTFVPPTVPVLLQILSGAQSAADLLPSGSVYALPSNATIELSLPAGALGGP-
3 TV Lac  -TNFFINDVSFVSPTVPVLLQILSGTTSAADLLPSGSLFALPSNSTIEISFPITATNAPG
             * ** * ** ************   * **** **   **** *** * *      *

1 PT Lac  --HPFHLHGHTFSVVQGAGQTVPNYVNPVRRDVVSIGAVPDNVTIRFETNNPGPWFLHCH
2 PR Lac  --HPFHLHGHTFSVVRPAGSTTYNYVNPVQRDVVSIGNTGDNVTIRFDTNNPGPWFLHCH
3 TV Lac  APHPFHLHGHTLSIVRTAGSTDTNFVNPVRRDVVNTGTAGDNVTIRFTTDNPGPWFLHCH
            ********* * *  ** *  * **** ****  *   ******* * **********

1 PT Lac  IDWHLDAGFAVVFAEDIPDIASVSPVPTDWSNLCPIYDALDPSDQ
2 PR Lac  IDWHLEAGFAVVFAEDIPDVASINPVPQDWSNLCPIYNALDASDH
3 TV Lac  IDFHLEAGFAIVFSEDTADVSNTTTPSTAWEDLCPTYNALDSSDL
          ** ** **** ** **  *          *  *** * *** **
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Fig. 3. Comparison of amino acid sequences of Plcc1 with other laccases of white-rot fungi. Identical amino acids from all three proteins are shown with the stars on the bottom line. Four possible copper-binding domains are shaded. Gaps are inserted for the optimal alignment and indicated by dashes. Accession numbers of sequences are as follows: PT Lac, laccase of *Phlebia tremellosa*, AM282562; PR Lac, laccase of *Phlebia radiata*, Q01679, showing 80.7% identity; TV Lac, laccase of *Trametes versicolor*, AM422387, showing 64.8% identity.

The addition of nonylphenol and other recalcitrant compounds in a liquid culture of *T. versicolor* greatly increased laccase activity (Mougin *et al*., 2002). During the degradation of synthetic lignin, not only MnP but also laccase was induced in *P. tremellosa* (Vares *et al*., 1994). We amplified a laccase cDNA fragment with the degenerated primers for the copper-binding domains, and this fragment was used for the full cDNA cloning by RACE-PCR. We successfully obtained the full cDNA (*plcc1*) from *P. tremellosa*, and this was reported to the EMBL nucleotide sequence database with the accession number AM282562. Plcc1 has the four copper-binding domains, which are highly conserved in other fungal laccases (Fig. 2). Plcc1 showed 80.7% and 64.8% identities when compared with *Phlebia radiata* (accession number Q01679) and *T. versicolor* (accession number AM42287), respectively (Fig. 3). Previously reported laccase from *P. tremellosa* 77-51 (Leontievsky *et al*., 1997) showed 75% identity in the first 20 N-terminal amino acid sequences compared with our Plcc1.

When *P. tremellosa* was grown with BBP (1 mM), BPA (0.2 mM) or DEP (1.8 mM) in a minimal medium, laccase activity was greatly increased with the addition of BBP and DEP, up to 26 times and 35 times, respectively, whereas no change was observed with the addition of BPA (Fig. 4a). Northern hybridization was performed in order to determine whether the laccase expression was parallel to the

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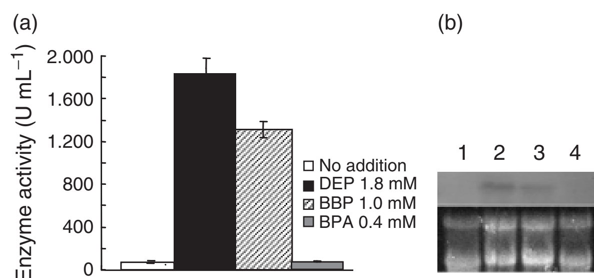

Fig. 4. Involvement of laccase in the degradation in *Phlebia tremellosa*. (a) Laccase activity in the culture supernatants of the minimal medium containing three different HMCs. (b) Confirmation of the laccase expression by Northern hybridization under the HMC-degrading conditions. Upper panel, determinations of the laccase transcripts from 4 different cultures; lower panel, photograph of RNA gel of the upper panel. Lane 1, control culture (no addition of HMC); lane 2, culture with DEP (1.8 mM); lane 3, culture with BBP (1 mM); lane 4, culture with BPA (0.2 mM). Laccase transcripts are shown only from the cultures containing BBP (lane 3) and DEP (lane 2).

activity when BBP, BPA and DEP were added to the culture medium. The induction of laccase expression was increased for both BBP and DEP addition, but no induction was observed for BPA addition, as in the case of the enzyme activity (Fig. 4b).

There are many reports concerning the laccase genes of white-rot fungi, because this enzyme is strongly implicated in the degradations of many diverse recalcitrant compounds, such as TNT and its catabolic intermediates (Han *et al.*, 2004; Cheong *et al.*, 2006), bisphenol A (Modaressi *et al.*, 2005), nonylphenol (Tsutsumi *et al.*, 2001) and herbicides (Rezende *et al.*, 2005; Maruyama *et al.*, 2006), and in dye decolourization (Rodriguez *et al.*, 1999). We tried to determine the laccase induction in complete media culture conditions because the natural environment is very complicated and is totally different from laboratory culture conditions. *Phlebia tremellosa* showed induced expression of laccase with the BBP or DEP in complete media as the results of the minimal medium culture conditions (data not shown). *Pleurotus ostreatus* also produced laccase when the fungus was cultured on basal medium containing a herbicide (Scepter) (Rezende *et al.*, 2005), and *T. versicolor* showed an induced laccase expression and activity when TNT and its catabolic intermediates were degraded (Cheong *et al.*, 2006).

In conclusion, *P. tremellosa* can degrade phthalic esters with reasonable rates, and the amounts of laccase transcript and the enzyme activity in this fungus are also enhanced by phthalic esters.

Acknowledgements

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