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ORIGINAL PAPER

Degradation of endocrine disrupting chemicals by genetic transformants in *Irpex lacteus* with an inducible laccase gene of *Phlebia tremellosa*

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Abstract *Irpex lacteus* was genetically transformed using an laccase expression vector to get increased laccase producing strains. Stable integration of the vector was confirmed by PCR using the vector-specific primers, and the transformants showed increased laccase activities. When the transformants were grown with several endocrine disrupting chemicals, laccase activity of each transformant was induced up to six times higher than that of the wild type. They showed increased degrading activities against EDCs as well as increased removal rates of estrogenic activities generated by the EDCs than the wild type, and the laccase expression was increased during the degradations of the EDCs.

Keywords Endocrine disrupting chemicals · Estrogenic activity · Laccase · White rot fungi

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Introduction

Various man-made chemicals are major pollutants in our environment, and disposable plastics cause serious problems during their incineration. There are certain chemicals which have been known to affect human reproductive systems, and the compounds mimicking or interfering with the action of endogenous gonadal steroid hormones have been named endocrine disrupting chemicals (EDCs; Colborn et al. 1996). Various EDCs are used in the plastic synthesis industry, and are also generated during the treatment of plastic wastes. Bisphenol A is widely used as a material for the production of epoxy and phenol resins, and lacquer coatings of food cans (Staples et al. 1998). Phthalates have been reported that they acquire unequivocal estrogenic activities by light exposure (Okamoto et al. 2006). EDCs interfere with animal reproductive system even at very low concentrations (Schonfelder et al. 2002). Since physical and chemical treatments for these EDCs can generate the secondary pollutants, biological treatments can be a clue. Even though they show slow removal rate, they do not require harmful chemicals unlike physical and chemical treatments.

White rot fungi have lignin degrading enzymes, such as laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP). These enzymes are also involved in the degradations of many recalcitrant chemicals such as dye (Faraco et al. 2009), EDCs (Cabana et al. 2007; Asgher et al. 2008), explosives (Cheong et al. 2006) and pesticides (Maruyama et al.

2006; Rezende et al. 2005). We have isolated many white rot fungi to examine their degrading activities against dyes, EDCs and other recalcitrant compounds such as polyaromatic hydrocarbons, and several fungi were selected by their degrading abilities against recalcitrant compounds. A white rot fungus *Irpex lacteus* has been used in the decolorization of dye (Shin 2004), and the degradations of explosive (Kim and Song 2003) and bisphenol A (Shin et al. 2007).

There are reports about the characterization of manganese peroxidase in this fungus (Shin et al. 2005; Baborova et al. 2006). However, laccase activity of this fungus is very low under various culture conditions (unpublished data) even though a mycelium-associated laccase has been reported (Svobodová et al. 2008).

A laccase activity has been reported to be greatly increased (26–35 times) during the degradation of phthalates in *Phlebia tremellosa*. We have cloned the laccase cDNA from *P. tremellosa* and confirmed its enhanced expression under the degrading conditions of phthalates (Yeo et al. 2008). We have determined its biochemical characteristics of the purified enzyme as well as the involvement of the enzyme in the removal of estrogenic activity generated by several EDCs (Kim et al. 2008a). We have also cloned the genomic DNA and promoter of the laccase (FM179742) from *P. tremellosa* using the cDNA as the probe. The promoter has one xenobiotic responsive element (GCGTG) and two metal responsive elements (GCRCNC). Genetic transformation by the restriction enzyme mediated integration (REMI) was performed, which cuts intergenic spaces in the expression vector in order not to disrupt important genes during the transformation of filamentous fungi (Leem et al. 1999). We have introduced the laccase expression vector into *I. lacteus* to generate enhanced degrading activity to several EDCs.

Materials and methods

Generation of laccase transformants showing higher laccase activity

Irpex lacteus isolated in Korea forest (Kim and Song 2003) was grown on potato dextrose agar at 30°C, and a minimal medium (Yeo et al. 2008) was also used when needed.

Genomic gene (3.1 kb) of a laccase from *P. tremellosa* was inserted at the *Bam*HI site of pBARGEM7-1 plasmid to construct a laccase expression vector (pBARPprolac), and this vector had the phosphinothricin resistance gene as the selectable marker. *I. lacteus* was transformed using the expression vector by following the previous report (Leem et al. 1999). Protoplasts (3×10^7/ml in 100 μl) were mixed with the vector (5 μg) and 30 μ of *Not*I to perform the REMI. Regenerated transformants were grown on the minimal medium plus phosphinothricin (100 μg/ml), and transferred to the same plate with higher concentrations of phosphinothricin (200 μg/ml) to confirm their resistance to phosphinothricin. Genomic DNAs from the recipient strain and the transformants were used as the template for PCR using two primers specific to the *trpC* promoter; 5′-GTCGACAGAAGATGATATT-3′ as the forward primer and bar; 5′-AGTTAGACAACCTGAAGTCT-3′ as the reverse primer for the confirmation of the genomic integration of pBARPprolac.

Degradation of EDCs and removal of estrogenic activities generated by the EDCs

Fungal strains were transferred to fresh liquid medium (100 ml) that contained one of the following EDCs (Aldrich) such as benzylbutylphthalate (BBP, 98%), bisphenol A (BPA, 99+%), diethylphthalate (DEP, 99.5%) or nonylphenol (NP, technical grade). The concentrations of the EDCs added to the medium were as follows: 300 mg/l (BBP), 100 mg/l (BPA), 400 mg/l (DEP) or 20 mg/l (NP). The culture supernatants were then collected regularly to measure the laccase and estrogenic activities. Fungal cells were also harvested, after which the expressions of the laccase gene were evaluated by real-time PCR using laccase gene-specific primers. Fungal cells (wet weight 5 g) harvested by centrifugation were extracted twice with 2 ml of phosphate buffer (50 mM, pH 6.8) to collect mycelium associated laccase, and the enzyme activity was measured. Total RNA was isolated from fungal cells obtained at different time periods using Trizol extraction buffer (Invitrogen) and the 3 μg of RNA from each culture was used as the template in the synthesis of cDNA. Real-time PCR was performed using 5 μl out of 20 μl from the cDNA mix, 12.5 μl of iTaq SYBR Green Supermix (BioRad), and 1 μl each laccase specific forward primer 5′-CATCCCTTCCA

CTTGCACG-3′, and reverse primer 5′-GATGTGGC AATGGAGGAACC-3′. The CT value of each sample was obtained using the ABI PRIAM 7000 SDS software, and the relative expression level of the laccase gene from each sample was then compared with the expression level of the actin gene. Laccase activity was measured by spectrophotometry using *o*-tolidine as the chromogenic enzyme substrate (Ko et al. 2001).

The residual concentrations of EDCs from culture supernatant and fungal cells were determined by Waters HPLC (HP 1,525 series, Gemini 5 μm C6-phenyl 110A 150 × 4.6 mm column) as reported previously (Kim et al. 2008b). The estrogenic activity was evaluated using the yeast two-hybrid system that has been previously described by Tsutsumi et al. (2001).

Results and discussion

Generation of laccase transformants induced by EDCs

More than 65 transformants were generated using the laccase expression vector in *I. lacteus*, and 2 transformants were selected to carry out further experiments. Two transformants (T7 and T8) produced laccase activity 2–3 times higher than the wild strain in the liquid minimal medium at day 3 (Fig. 1a). Fungal cells were extracted with phosphate buffer to assay the mycelium associated laccase activities from the wild type and the transformants, and very low activities (14–24 U/g mycelium) were detected from the three strains. This result meant that the wild type strain was genetically transformed to high laccase-secreting transformants. These strains also induced and secreted high amount of laccase activity in the liquid media containing four different EDCs (Fig. 1b). Four different EDCs were added to the cultures to obtain linear growth rates that were similar between all cultures evaluated here (ca. 70% growth of control culture). Laccase activity of *P. tremellosa* was increased up to 26–35 times in the liquid media by addition of phthalates (Yeo et al. 2008). Even though two transformants carried the same laccase promoter and gene from *P. tremellosa*, none of the transformants showed such a high induction; the enzyme activity was increased about twice by the addition of each phthalate, however,

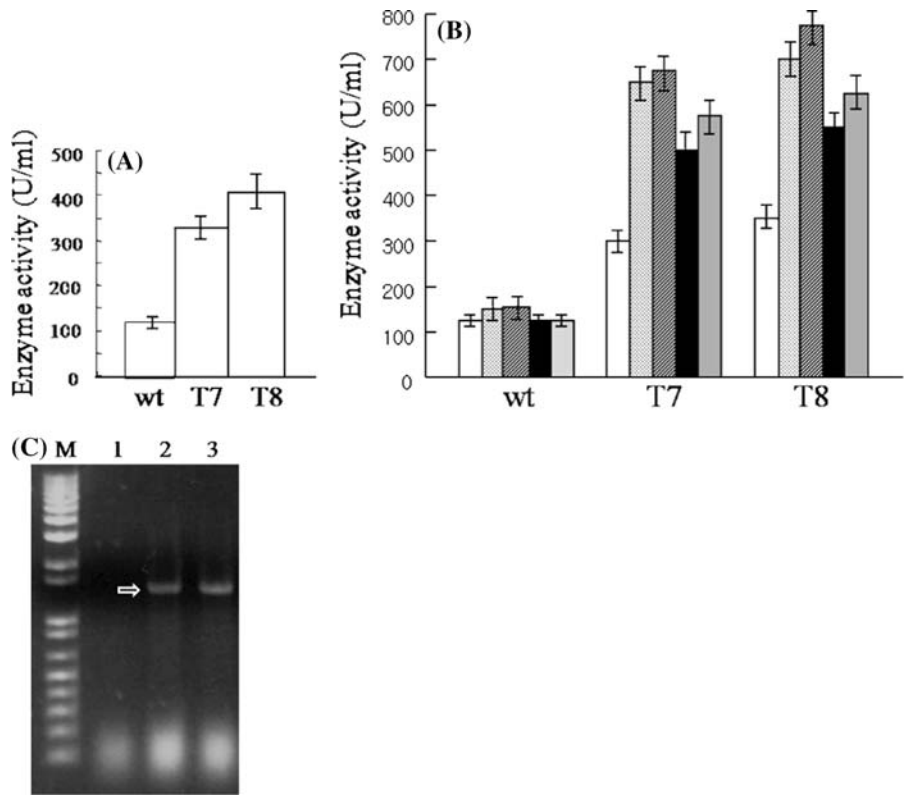

Fig. 1 **a** Extracellular laccase activity of each fungal strain in the minimal liquid medium at day 3. **b** Increase of laccase activity at day 3 during the degradation of EDCs from each fungal strain. *White bar* control culture (no addition of EDC); *dotted bar* BBP (300 mg/l); *hatched bar* DEP (400 mg/l); *black bar* BPA (100 mg/l); *gray bar* NP (20 mg/l). **c** Confirmation of the integration of the expression vector into the transformant chromosomal DNA. *M* molecular weight marker; *1* recipient strain; *2* transformant 7; *3* transformant 8. *White arrow* amplified DNA fragment

they showed 5–6 times higher enzyme activities when compared with the wild type. Furthermore, the transformants also showed increased laccase activities by the addition of BPA (4–6 times) or NP (5.5–6.5 times; Fig. 1b). The stable integration of the laccase expression vector in the transformants was examined by PCR using vector-specific primers, and the amplified DNA fragment appeared from the total DNA of two transformants (Fig. 1c).

Degradation of EDCs and removal of estrogenic activities generated by the EDCs

Each EDC in the culture broth of transformants and the wild type was degraded rapidly, which was determined by HPLC. The degradation of BBP and DEP was more than 90% in both wild type and transformants on day 3 (Fig. 2a), even though the initial degradation rates for two EDCs were faster in transformants (data not shown). However, the degradation of BPA and NP were slower than those of BBP and DEP, but two transformants showed higher degradation rates for these two EDCs than the wild type (Fig. 2a). The degradation of BPA on day 3 by T8 (ca. 55%) was lower than the previous result, which 5-day pre-incubated culture with 50 mg/l reached almost 100% degradation of the original compound (Shin et al. 2007). We have added the chemicals with the inoculums, and added BPA by 100 mg/l instead of 50 mg/l, which resulted in such difference. When the estrogenic activity generated by BPA was examined using the yeast two-hybrid system, more than 70% of estrogenic activity was removed by two transformants while only 30% was removed by the wild type strain (Fig. 2b). In case of NP, more than 80% of estrogenic activity was removed by the transformants while the wild type removed much less than the transformants (<20%, Fig. 2b). The transformants also showed increased removal rates of estrogenic activity than the wild type towards phthalates (Fig. 2b).

Laccase expressions during the degradation of EDCs were determined by quantitative real-time PCR using the laccase gene-specific primers, and transformant 7 (column 2, 5, 8) and transformant 8 (column 3, 6, 9) showed highly increased expression of laccase under degrading conditions of BBP (column 4–6) and DEP (column 7–9) than the recipient strain (column 1, 4, 7; Fig. 2c; column

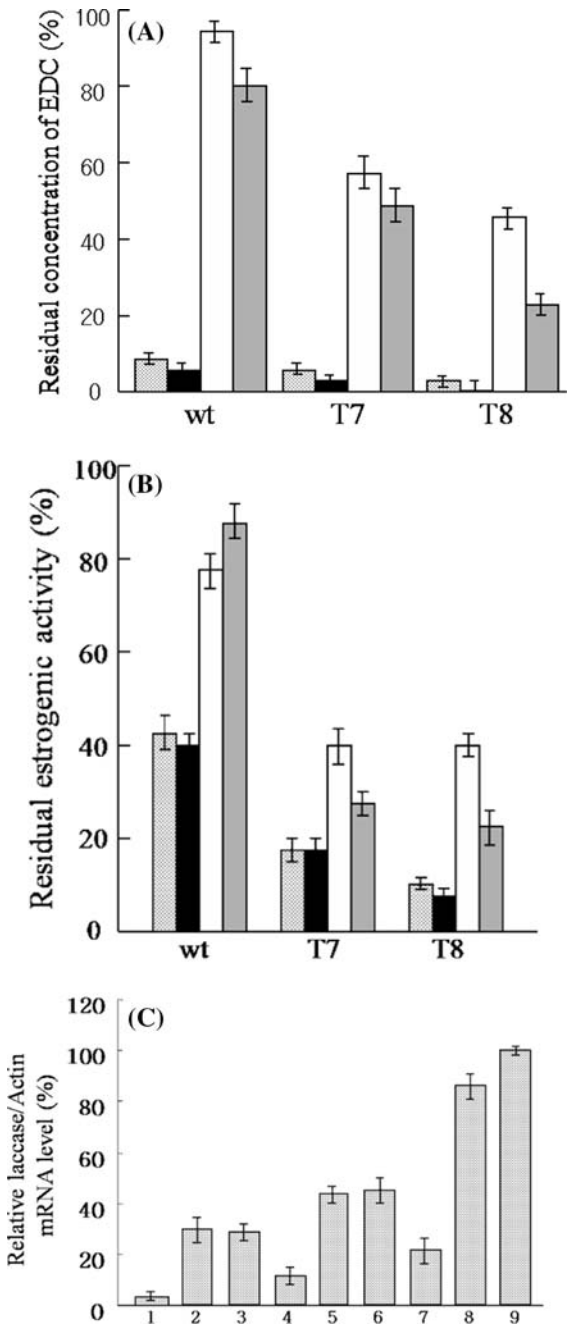

Fig. 2 **a** Determination of concentrations of EDCs in the cultures at day 3 by HPLC. *Dotted bar* BBP; *black bar* DEP; *white bar* BPA; *gray bar* NP. **b** Residual estrogenic activity generated by each EDC at day 3. Legend was same as in Fig. 2a. **c** Comparison of the laccase expression by real-time PCR using the laccase gene-specific primers at day 3. *1*, *4*, *7* recipient strain; *2*, *5*, *8* transformant 7; *3*, *6*, *9* transformant 8. *1–3* control culture (no EDC); *4–6*, BBP; *7–9*, DEP

1–3, no EDC). This was an expected result because laccase expression was detected during the degradation of phthalates in *P. tremellosa* (Yeo et al. 2008). Since increased MnP expression was reported in *Trametes versicolor* under the EDC degrading conditions (Kim et al. 2008b), MnP and/or LiP must also be involved in degrading the EDCs in these transformants. The increased laccase activities in the transformants seemed to be deeply implicated in the degradation of the EDCs and the removal of the estrogenic activities generated by the EDCs, because the laccase enzyme from *P. tremellosa* was positively involved in the removal of the estrogenic activity generated by several EDCs (Kim et al. 2008a). This increase of laccase activity by BPA addition was not observed in *P. tremellosa* (Yeo et al. 2008), therefore two transformants could be good candidates for the bioremediation of phthalates as well as BPA and NP. A fungus which has all of the three lignin degrading enzyme activities can be useful for the degradation of EDCs, and the transformants show another possibility for the degradation of recalcitrant compounds.

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