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Phytoremediation of Bis-Phenol A via Secretory Fungal Peroxidases Produced by Transgenic Plants

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1. Introduction

The fungal lignin-degrading enzymes lignin peroxidase (LiP, E.C. 1.11.1.14), Mn-dependent peroxidase (MnP, E.C. 1.11.1.13), and phenol oxidase (laccase) (Lac, E.C. 1.10.3.2) can degrade or polymerize organic pollutants such as polychlorophenols, polycyclic aromatic hydrocarbons, and chlorinated hydrocarbons (Fernando and Aust, 1994; Hammel, 1989; Hirano et al., 2000; Levin et al., 2003; Lin et al., 1990; Lovley et al., 1994; Mohn and Tiedje, 1992; Reddyy et al., 1998). However, to maintain such fungal lignin-degrading enzymes at adequate levels for degradation or detoxification (bioremediation), appropriate additions of both microorganisms and nutrients are essential over long periods of time. Recently, phytoremediation technology has gained attention for its potential as an ecological remediation tool of contaminated soil and water, as plants can grow autotrophically. Establishment of effective phytoremediation technology is a suitable strategy for the long-term remediation of contaminated areas. Phytoremediation includes some processes based on the plant functions as follows; phytostabilization, which is accumulation of pollutants in the rhizosphere by absorption on the root surface, precipitation, and complexation of pollutants; rhizodegradation, which is degradation of pollutants by interaction with rhizosphere microorganisms; phytoaccumulation (phytoextraction), which is uptake and accumulation of pollutants by plants; phytodegradation (phytotransformation), which is uptake and degradation of pollutants by plants; and phytovolatilization, which is uptake and volatilization of pollutants by transpiration from contaminated area. To widely apply the benefit of phytoremediation, improvement and reinforcement of the abilities for uptake, accumulation and degradation of pollutants using genetic engineering are one of the important development subjects.

There have been many reports of phytoremediation using transgenic plants. For example, glutathione S transferase and cytochrome P450 expression showed high resistance to pesticides (Gullner et al., 2001; Doty et al., 2000), the overexpression of bacterial mercury

reductase showed high resistance to organic mercury (Bizilly et al., 2003) and effective volatilization of ionic mercury (Haque et al., 2010), pentaerythritol tetranitrate reductase-expressing plants were able to degrade glycerol trinitrate and 2,4,6-trinitrotoluene (French et al., 1999), introduction of bacterial genes involved in polychlorinated biphenyl (PCB) degradation in plants showed removal of PCB from a contaminated area (Novakova et al., 2009), the bacterial arsenite S-adenosylmethyltransferase expression induced arsenic methylation and volatilization (Xiang-Yan et al., 2011), the expression of gamma-glutamylcysteine synthetase and the genes involved in phytochelatin synthesis in plant showed more resistance and accumulation of cadmium (Zhu et al, 1999, Wawrzyński et al, 2006), and the yeast metallothionein expressing tobacco showed effective copper uptake (Thomas et al, 2003).

Recently, attempts are carried out to enhance the environmental remediation in contaminated area by using appropriate genetically modified plants with usage of fungal peroxidases. This chapter mainly focused on the removal of bis-phenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane), which is one of the major chemicals used in plastics and resins and is well known to disrupt endocrine systems in humans and other animals, from contaminated areas with usage of transgenic technology. Although many organisms can degrade and metabolize BPA, which can lead to a reduction of the estrogenicity and toxicity of BPA (Kang et al., 2006), lignin-degrading basidiomycete fungi are particularly powerful degraders of organic pollutants including BPA. These fungi produce oxidative enzymes, such as LiP, Lac, and MnP, which can degrade and polymerize BPA both *in vivo* and *in vitro*. Therefore, an overview of our recent results regarding the phytoremediation of BPA with fungal peroxidase-expressing transgenic plants by lignin peroxidase (LiP), laccase (Lac), and manganese peroxidase (MnP) were presented together with the other potential uses of these transgenic plants in this chapter.

2. LiP-expressing transgenic tobacco

cDNA (Accession no. AB158478.1) encoding LiP from the reverse transcription (RT) products of total RNA prepared from mycelia of *Trametes versicolor* IFO1030 was isolated. The cloned cDNA was ligated into binary vector pBI121 (Brasileiro et al., 1991) with double cauliflower mosaic virus (CaMV) 35S promoter sequence (Figure 1), and was introduced into the genome of the tobacco (*Nicotiana tabacum* Samsun NN) by the leaf-disk method via *Agrobacterium tumefacience* LBA4404 (Liang et al., 1989).

Integration of the cDNA into the genome of tobacco was confirmed by polymerase chain reaction (PCR) upon 10 independent transgenic lines. Two of the lines showed growth inhibition and thus were excluded from further analysis. Western blot analysis with root extracts of transgenic tobaccos and antiserum raised against LiP protein were performed to confirm the production of LiP protein in roots of transgenic lines. . To prepare the antiserum against LiP of *T. versicolor* IFO1030, we synthesized one peptide, whose sequence was ^{240}CNGTTFPGTGDNQG^{254}E, and conjugated it with keyhole limpet hemocyanin (KLH). The resultant peptide-KLH conjugant was injected into a 10-wk-old rabbit. After four injections, antiserum was collected and used for Western blot analysis. The expected signal was observed in the cell-free extracts of roots from LiP transgenic tobaccos (Figure 2).

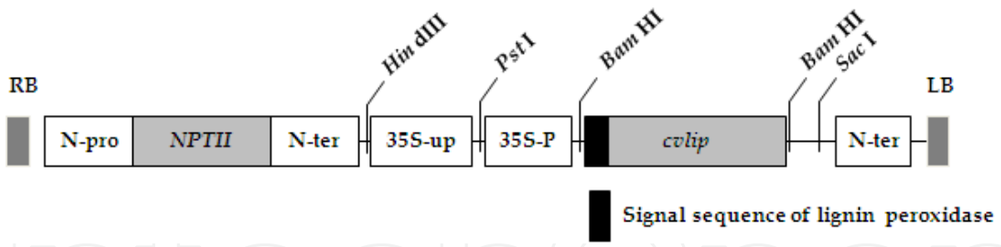

Fig. 1. Gene construct of T-DNA region of Ti plasmid.

RB, Right border of T-DNA; N-pro, promoter region of nopaline synthase gene; *NPTII*, neomycin phosphotransferase gene; N-ter, terminator region of nopaline synthase gene; 35S-up, upstream region of cauliflower mosaic virus (CaMV) 35S promoter sequence; 35S-P, CaMV 35S promoter sequence; *cvlip*, cDNA encoding LiP of *T. versicolor* IFO1030 plus signal sequence; LB, left border of T-DNA.

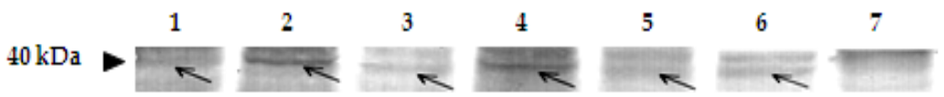

Fig. 2. Western blot analysis of LiP in cell-free extracts of roots of LiP transgenic lines.

Lanes; 1, LiP transgenic line (FLP)-1; 2, FLP-2; 3, FLP-3; 4, FLP-4; 5, FLP-5; 6, FLP-8; 7, control plant.

To test the ability of BPA removal by LiP-expressing transgenic plants, we transferred two-month-old transgenic lines on MS medium (Murashige and Skoog, 1962) to fresh MS liquid medium containing 3 g/L of glucose and 100 µg/L of kanamycin. After one week of incubation at 25°C, BPA was added to the medium at the final concentration of 100 µM and the medium was hydroponically incubated for another week. The six LiP-expressing transgenic lines showed 2- to 4-fold higher BPA removal ability than that of control plants during aqueous cultivation (Figure 3). LiP is a well-known enzyme that carries out direct and indirect oxidation of a number of environmental pollutants. Our confirmation that transgenic plants could express LiP in their roots and remove BPA will help us to establish improved methods for phytoremediation of contaminated environments.

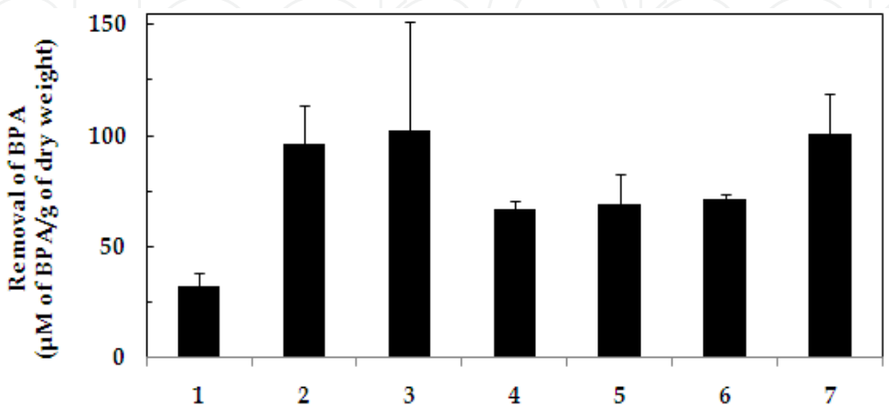

Fig. 3. Removal of BPA by LiP-expressing transgenic lines.

The levels of BPA were analyzed by HPLC (λ=278 nm). The values shown are the average of results from three independent experiments. Lanes; 1, control; 2, FLP-1; 3, FLP-2; 4, FLP-3; 5, FLP-4; 6, FLP-5; 7, FLP-8. Error bars on the graph indicate standard deviations (*N*=3).

3. Lac-expressing transgenic tobacco

Lac is a member of the multicopper oxidase family found in a wide range of organisms such as animals, plants, bacteria, and fungi. The reduction of oxygen to water is accompanied by the oxidation of substrate by laccase.

cDNA encoding Lac (Accession no. D13372.1) from the reverse transcription products of total RNA prepared from mycelia of *T. versicolor* IFO1030 was cloned. The cDNA under the control of double CaMV 35S promoter was introduced into the genome of *N. tabacum* Samsun NN by the leaf-disk method via *A. tumefacience* LBA4404 (Figure 4).

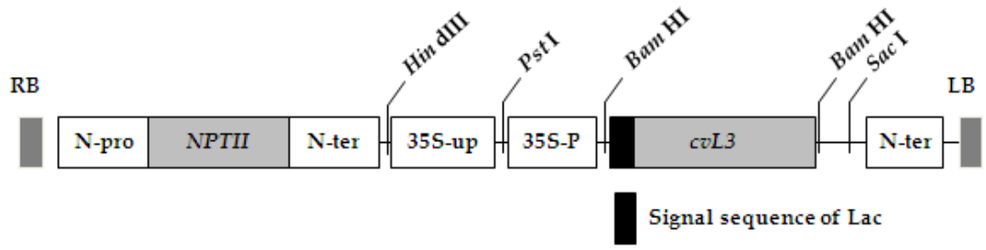

Fig. 4. Gene construct of T-DNA region of Ti plasmid.

cvL3, cDNA encoding Lac of *T. versicolor* IFO1030 plus signal sequence. Other abbreviations are listed in Figure 1.

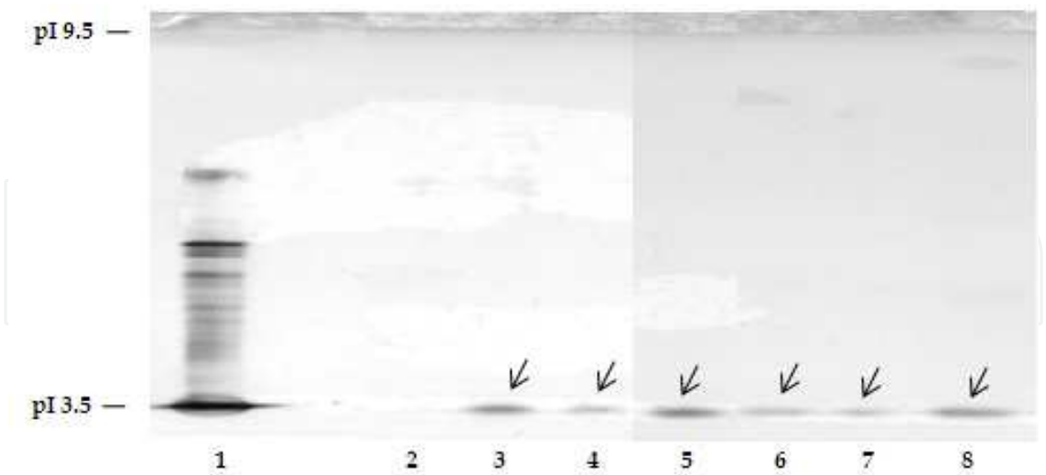

Fig. 5. Active staining of secreted Lac from the roots of transgenic lines.

Concentrated 60 μg of crude extracellular protein was analyzed by IEF and active staining using 4-chloro-1-naphthol. Lanes, 1, Concentrated aqueous cultivation medium of *T. versicolor* IFO1030; 2, control; 3, Lac transgenic line (FL)-4; 4, FL-5; 5, FL-9; 6, FL-20; 7, FL-22; 8, FL-23.

Two-month-old transgenic lines, which were incubated on MS medium, were transferred to fresh MS liquid medium and subjected to further incubation. After two weeks, to confirm the expression of Lac protein and secretion from the roots of each transgenic line into the rhizosphere, we concentrated the aqueous culture medium and analyzed it by iso-electric focusing electrophoresis (IEF) and active staining using 4-chloro-1-naphtol (Figure 5). Six independent transgenic lines apparently secreted active Lac protein into their rhizosphere, and we tested four of those to determine their ability to remove BPA. As described above, four independent transgenic lines were cultivated hydroponically. After one week of incubation, BPA was added to the medium at the final concentration of 100 μM and hydroponic incubation was done for another week. The ability to remove BPA of these Lac-expressing transgenic tobaccos was more than 5-fold that of the control line during hydroponic cultivation (Figure 6).

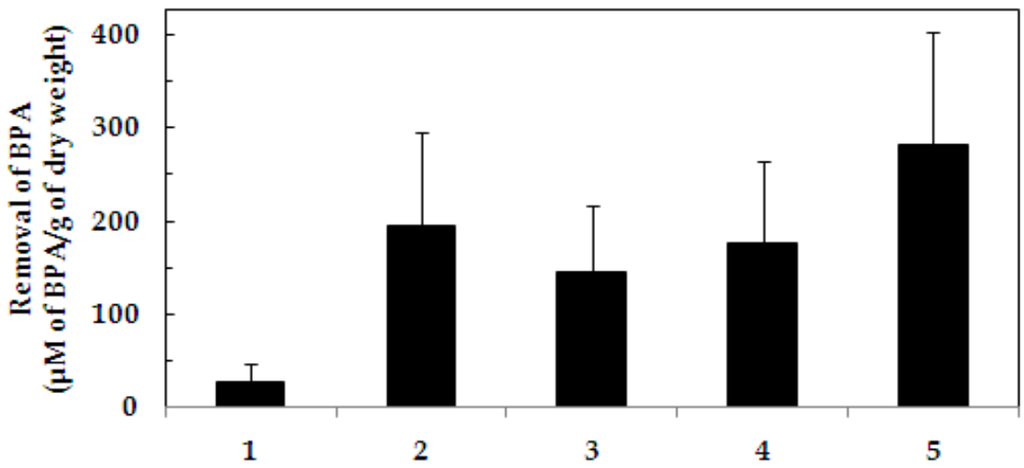

Fig. 6. BPA removal ability of Lac transgenic lines.

The levels of BPA were analyzed by HPLC (λ=278 nm). The results shown are the average of three independent experiments. Lanes; 1, control; 2, FL-4; 3, FL-9; 4, FL-20; 5, FL-22. Error bars on the graph indicate standard deviations (*N*=3).

All of these Lac-expressing transgenic tobaccos were somewhat shorter than control plants at the flowering stage, and most of the transgenic anthers failed to dehisce after blooming, while the anthers of control plants were normally dehiscent (Figure 7). In addition, the nondehiscent anthers were brown in contrast to the greenish control lines. Brown pigmentation and rough epidermis were observed on the surface of transgenic anthers. Greater Lac activity was detected in the cell-free extracts of transgenic anthers than in the controls; however, there was no correlation with lignin contents in transgenic anthers (Figure 8). Histochemical analysis of anther tissues revealed apparent deformation of the stomium in transgenic plants (Figure 9). Beals reported that the stomium in anther tissue plays a crucial role in the dehiscence of anthers in tobacco (Beals, 1997), indicating that such deformation of stomium observed in the transgenic anther tissue might affect the appearance of the nondehiscent phenotype. The expression of Lac could promote the efficient removal of BPA, but it also influences some aspects of flower development.

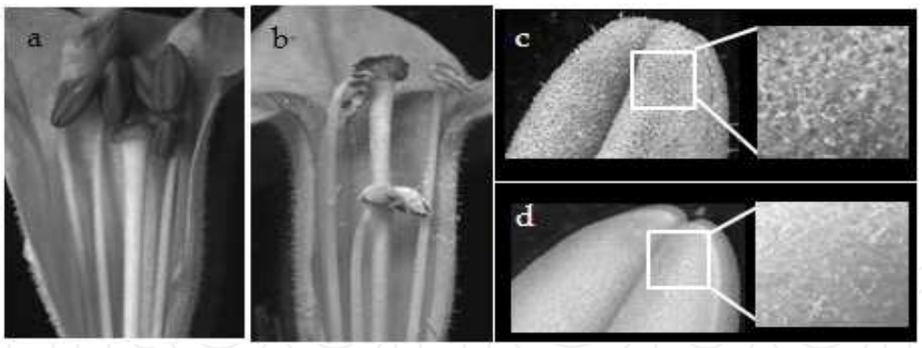

Fig. 7. Phenotypes of anthers of Lac transgenic lines.

Transgenic and control tobaccos were cultivated at 24°C. a, Transgenic flower with nondehiscent anthers. b, Control flower with normal anthers. c, Stereomicroscopic view of a transgenic anther. d, Stereomicroscopic view of a normal anther.

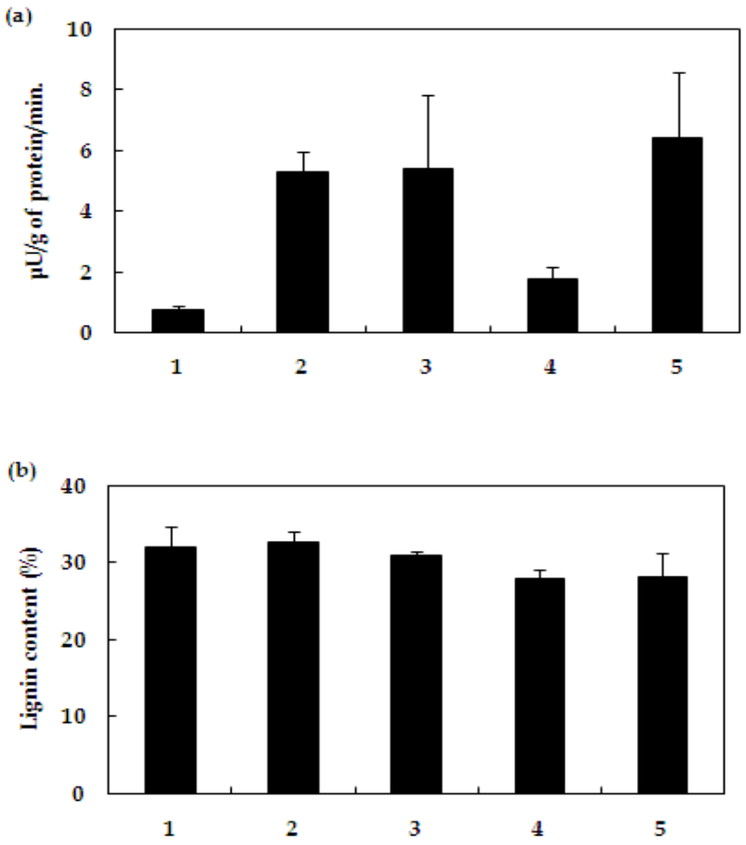

Fig. 8. Laccase activity and lignin content in anther tissues.

Transgenic and control tobaccos were cultivated at 24°C. a, Laccase activity. Cell-free extracts were prepared from both transgenic and control anthers before they dehisced. Laccase activity was calculated using the extinction coefficient (6400 $M^{-1}cm^{-1}$) of oxidized guaiacol (λ=436nm), and activity was expressed as definitive units (1 unit = 1 mol guaiacol

oxidized per min) (Eggert et al, 1996). b, Lignin content. Lignin was quantified by the Klason method. The results shown are the average of three independent experiments. Error bars on the graph indicate standard deviations (*N*=3). Lanes; 1, control; 2, FL4; 3, FL9; 4, FL20; 5, FL22

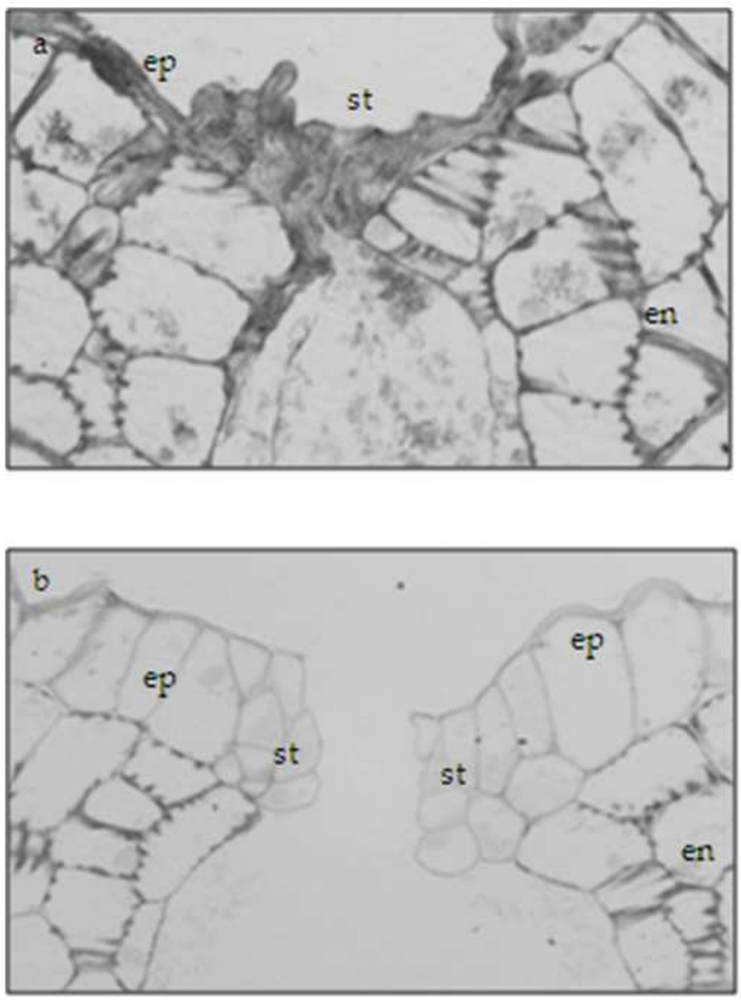

Fig. 9. Histochemical analysis of anther tissues.

Safranin-stained thin sections of a mature anther from a transgenic (a) and a control plant (b). ep, epidermis; st, stomium; en, endothecium cell.

4. MnP-expressing transgenic hybrid aspen

MnP is a heme peroxidase that can oxidize phenolic compounds in the presence of Mn (II) and hydrogen peroxide. Mn (II) is oxidized to Mn (III) by MnP; the resultant Mn (III) makes a chelating compound with an organic acid, and then organic compounds such as BPA are oxidized by the chelating compound. Previously, we isolated a cDNA (Accession no. AR429405) encoding MnP from *T. versicolor* and introduced it into the genome of *N. tabacum* Samsun NN. The transgenic tobacco could express MnP and produce Mn (III) as a result of Mn (II) oxidation in the rhizosphere during hydroponic cultivation (Iimura et al., 2002).

Moreover, isolated cDNA was also introduced into the genome of hybrid aspen Y63 (*Populus seiboldii* x *Populus gradientata*) under the control of double CaMV 35S promoter (Figure 10), as described previously (Kajita et al., 2004). Integration of the T-DNA into the genome of each transgenic line was confirmed by PCR. Although the expression of cDNA encoding MnP was confirmed by RT-PCR in six independent transgenic lines, MnP activity was detected in four of the six lines (Figure 11). The BPA-removing activities of the four MnP-expressing transgenic hybrid aspens were more than twice that of the control lines (Figure 12). Interestingly, the expression of the MnP gene showed no phenotypical differences between the MnP-expressing and control plants, unlike the expressions of the LiP and Lac genes. The lack of negative effects of MnP expression on growth and development will be advantageous when it is used in phytoremediation. Our results showed that the transgenic plants could express MnP in their roots and contribute to the effective removal of BPA from a hydroponic medium.

Fig. 10. Gene construct of T-DNA region of Ti plasmid.

fmnp, cDNA encoding MnP of *T. versicolor* IFO1030 plus signal sequence. Other abbreviations are listed in Figure 1.

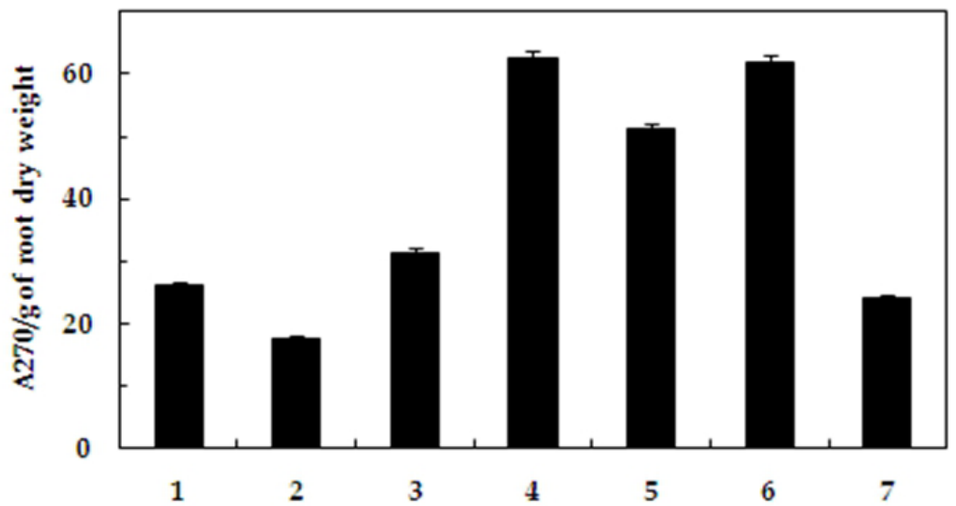

Fig. 11. MnP activity in root exudates of transgenic lines. Undamaged root tissues were dipped in 50 mM malonate buffer (pH 4.5) containing 1 mM manganese sulfate. After incubation for 24 hrs at 37°C, the absorbance of supernatant was measured at 270 nm. Lanes; 1, control plant; 2, MnP transgenic line (FM)-1; 3, FM-2; 4, FM-3; 5, FM-4; 6, FM-7; 7, FM-8. Error bars on the graph indicate standard deviations (*N*=3).

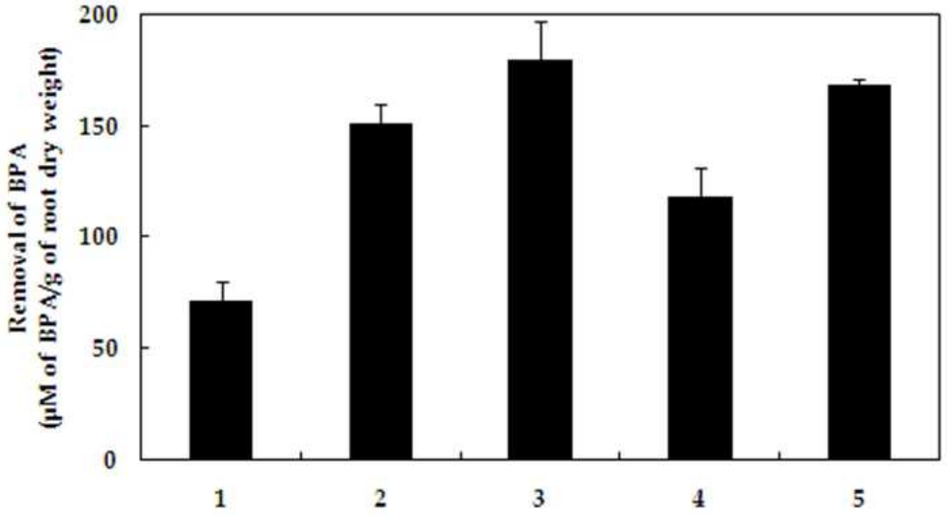

Fig. 12. BPA removal ability of MnP transgenic lines.

The levels of BPA were analyzed by HPLC (λ=278 nm). The values shown are the average of results from three independent experiments. Lanes; 1, control; 2, FM-2; 3, FM-3; 4, FM-4; 5, FM-7. Error bars on the graph indicate standard deviations (*N*=3).

As described above, fungal peroxidase (LiP, Lac, and MnP)-expressing transgenic plants showed effective BPA removal ability, but no reaction products of BPA conversions by these fungal peroxidase-expressing transgenic plants were detected under our analytical conditions. The enzymatic reaction of fungal peroxidases is non-specific and free radical-based, so it is difficult to detect the reaction products. BPA might be degraded or polymerized, as reported in some previous studies of lignolytic enzymes (Hirano et al., 2000; Fukuda et al., 2001; Tsutsumi et al., 2001; Uchida et al., 2001). The increase of BPA removal efficiency by the fungal peroxidase expression in plants would contribute to the development of remediation systems for the cleanup of contaminated areas.

5. Conclusions

Plants can metabolize BPA. Cultured cells of plants were able to glucosylate BPA (Nakajima et al., 2002; Hamada et al., 2002), and, in seedlings, BPA was absorbed from roots and translocated to leaves after glucosylation (Nakajima et al., 2002). In addition, some glycosylated forms of BPA showed less estrogenic activity than that of non-glycosylated BPA (Morohoshi et al., 2003), and oxidative enzymes in plants such as peroxidases stimulated the degradation and polymerization of BPA (Sakuyama et al., 2003). Although the ability of plants to detoxify might be useful for remediation of soil and water contaminated with BPA, the expressions of fungal peroxidases in plants by genetic engineering, as reviewed above, reinforces their ability with respect to the detoxification of BPA. Furthermore it is worth noting that the MnP- and Lac-expressing transgenic plants could remove pentachlorophenol effectively from contaminated areas during hydroponic cultivation (Iimura et al., 2002; Sonoki et al., 2005). Plants could secrete Lac and generate Mn (III) in the rhizosphere, and then the Lac and Mn (III) might be able to affect hydrophobic substrates, such as pentachlorophenol, which is difficult for plant roots to absorb.

Plants producing fungal secretory peroxidases would provide us useful tools for the remediation of areas contaminated with environmental pollutants. Further studies on the effective expression and the secretion of introduced enzymes and the application with other substrates will play an important role in the development of phytoremediation technology.

6. Acknowledgement

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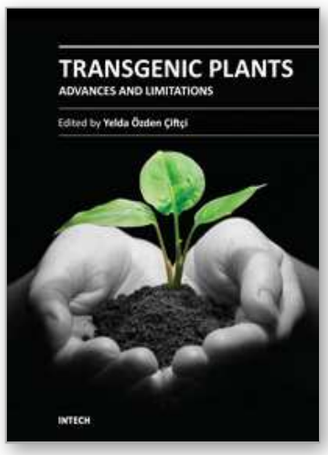

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