J Wood Sci (2007) 53:541–544
DOI 10.1007/s10086-007-0890-z
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Hybrid aspen with a transgene for fungal manganese peroxidase is a potential contributor to phytoremediation of the environment contaminated with bisphenol A

Received: October 18, 2006 / Accepted: February 13, 2007 / Published online: May 31, 2007

Abstract To assess the possible utility of a fungal gene for manganese-dependent peroxidase (MnP) produced by a transgenic plant in phytoremediation, we transformed hybrid aspen with a chimeric gene for MnP. Our gene construct allowed expression of the gene for MnP in plants and relatively high MnP activity was detected in the hydroponic medium in which roots of plants that expressed the transgene had been cultured. Some of our transgenic plants were able to remove bisphenol A from the medium more efficiently than wild-type plants. Our results demonstrate that, without any modification of the coding sequence, a chimeric gene for fungal MnP can be expressed in a woody plant, with secretion of active MnP from roots into the rhizosphere. Our strategy suggests new options using woody plants for phytoremediation.

Key words Bisphenol A · Manganese-dependent peroxidase · Phytoremediation · *Populus* · *Trametes versicolor*

Introduction

Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane], an endocrine disrupter, is known to be a xenoestrogen and to affect the reproductive functions of animals. However, the evidence for a low-dose effect of BPA remains controversial (for review, see Staples et al.[1]). BPA is used for the production of a variety of polymers, such as polycarbonates, polyesters, and polyacrylates. It is released from such polymers when they are washed, heated, or exposed to acid. Because of the endocrine-disrupting effect of BPA, it is possible that industrial discharge of BPA into the environment might be the cause of serious environmental problems.

Many organisms can degrade and metabolize BPA, contributing to a reduction in the estrogenicity and toxicity of this compound (for review, see Kang et al.[2]). In particular, lignin-degrading basidiomycete fungi are powerful degraders of BPA. These fungi produce oxidative enzymes, such as manganese-dependent peroxidase (MnP) and laccase, which can degrade and polymerize BPA both in vivo and in vitro. 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. Hirano et al.[3] reported that MnP, purified from *Pleurotus ostreatus*, converted BPA to compounds such as phenol and 4-isopropenylphenol. Tsutsumi et al.[4] reported that MnP from *Phanerochaete chrysosporium* decreased both the concentration of BPA in a contaminated reaction mixture and the estrogenic activity of BPA. These results suggest that the fungal MnPs might be powerful tools for removal of BPA from the environment.

In addition to fungi, plants can metabolize BPA both in vivo and in vitro. Cultured cells and seedlings of tobacco plants can convert BPA to sugar-conjugated forms.[5] In tobacco seedlings, BPA was absorbed via the roots, metabolized in internal tissues to its β-glucoside and then transported, as the β-glucoside, to leaves. Hamada et al.[6] also reported the glycosylation of BPA by cultured cells of *Eucalyptus perriniana*. Some glycosylated forms of BPA have lower estrogenic activity than BPA itself.[7] Furthermore, plant oxidative enzymes, such as peroxidases and polyphenol oxidases, also contribute to the degradation and polymerization of BPA.[8] The ability of plants to detoxify BPA might be useful for remediation of soil and water that have been contaminated with this compound.

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Phytoremediation has attracted attention as an ecologically sound technology for the remediation of contaminated soil and water. Plants can grow autotrophically; thus, phytoremediation is a suitable strategy for the continuous remediation and maintenance of large contaminated areas. In particular, trees such as aspen and poplar can reach and remediate contaminated soil and water at greater depths than herbaceous plants. Moreover, trees are perennial and can process large amounts of water through transpiration. Transgenic and nontransgenic *Populus* trees have already been used for the phytoremediation of soil contaminated with organic compounds such as atrazine,[9] trichloroethylene,[10] and 1,4-dioxane.[11]

We reported previously that MnP-expressing transgenic tobacco plants were able to secrete MnP from their root systems and remove pentachlorophenol effectively from a hydroponic medium.[12] In this study, we applied the MnP-production system that we established in transgenic tobacco to hybrid aspen. To our knowledge, this is the first report of transgenic hybrid aspen trees that express a chimeric gene for MnP. We discuss the expression of the gene for MnP, the activity of MnP in the plants, and the ability of plants to remove BPA from the growth medium.

Materials and methods

Generation of transgenic hybrid aspen

Hybrid aspen Y63 (*Populus seiboldii* × *Populus gradientata*) was transformed by the previously reported protocol.[13] Stem and leaf segments of plants were inoculated with *Agrobacterium tumefaciens* LBA4404 that harbored pW35SfMnP, of which the T-DNA region included expression cassettes for MnP from *Trametes versicolor* (GenBank accession no. AR429405) and for a selection marker (kanamycin-resistance gene). As described in our previous report, this plasmid, pW35SfMnP, was also used for production of transgenic tobacco plants with the fugal MnP gene.[12] Regenerated shoots from each segment that grew on kanamycin-containing medium (100 mg/l) were rooted and maintained on modified Murashige-Skoog (MS) medium supplemented with 0.05 mg/l indole-3-butyric acid and 100 mg/l kanamycin. The resultant young plants were cultured in glass vessels or bottles under sterile conditions.

Analysis of transgenic plants by PCR and RT-PCR

Integration of T-DNA into the genome of each transgenic line was confirmed by the polymerase chain reaction (PCR). The fragment corresponding to the introduced gene for MnP was amplified with total DNA isolated from each line as template and primers, cvmnpF (5′-GGCATCTCTCCTTC CATCGCCT-3′) and cvmnpR (5′-TTGAACGCGGACT GGAGCTTGG-3′). A DNA fragment of 648 bp is amplified under these conditions if total DNA includes an integrated gene for MnP. Using reverse transcription and PCR (RT-PCR), we also examined the expression of the gene for MnP in each transgenic line. Total RNA was extracted from roots of each transgenic line with the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) and then first-strand cDNA was synthesized from the resultant RNA (0.8 μg) after treatment of the RNA with RNase-free deoxyribonuclease. Both PCR and RT-PCR were performed with the same set of primers and under the following conditions: preincubation at 95°C for 10 min; then 25 to 35 cycles of incubation at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 30 s; final extension at 72°C for 5 min.

Detection of secreted MnP in hydroponic medium

The MnP activity in root exudates was measured as described in a previous report.[12] In brief, root tissue (100 mg fresh weight) from each transgenic plant that had grown to about 20 cm in height was cut from the stem and transferred to a microtube that contained 100 μl of 50 mM malonate buffer (pH 4.5) plus manganese sulfate (1 mM). After a 24-h incubation at 37°C, the supernatant of the reaction mixture was diluted by addition of 400 μl of 50 mM malonate buffer (pH 4.5). Absorbance of the supernatant was measured at 270 nm with malonate buffer supplemented with manganese sulfate as the reference sample. The activity was expressed as absorbance per gram of root tissue.

Removal of BPA from hydroponic medium by MnP-producing hybrid aspen

We measured the reduction in level of BPA in hydroponic medium by transgenic plants as described previously.[12] Transgenic plants, growing on MS-agar medium, were transferred to 100-ml aliquots of fresh MS liquid medium that contained 3 g/l sucrose and 100 mg/l kanamycin. After further incubation for 1 week at 25°C, BPA was added to the medium at a final concentration of 100 μM and plants were maintained in hydroponic culture for another week. A 1-ml aliquot of the hydroponic culture medium was then centrifuged at 10 000 *g* for 5 min and a 100-μl aliquot of the supernatant was subjected to analysis by high-performance liquid chromatography (HPLC). Chromatographic separations were performed with a C18 cartridge column (length, 150 mm; i.d., 4.6 mm) with a mobile phase that consisted of a mixture of 0.2% phosphoric acid and acetonitrile (65:35, v/v) and a flow rate of 1.0 ml/min. The eluate was monitored with an ultraviolet (UV) detector at 278 nm.

Results and discussion

We introduced a full-length cDNA of 1098 bp that encoded MnP from *Trametes versicolor*, under the control of a double 35S promoter sequence from cauliflower mosaic virus, into the genome of hybrid aspen by *Agrobacterium*-mediated transformation. After screening, we obtained seven putative transgenic plants. In order to confirm the integration of the transgene into the genome of these plants,

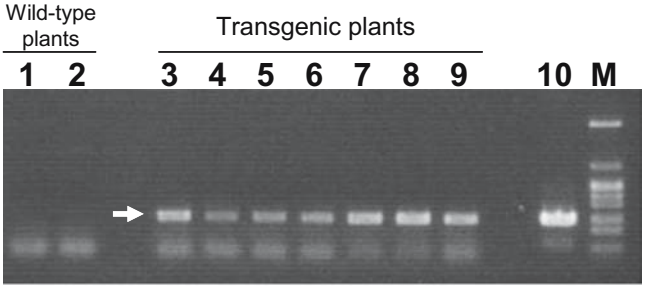

Fig. 1. Analysis by polymerase chain reaction (PCR) of total DNA from wild-type and transgenic hybrid aspen. Amplified fragments (648 bp; indicated by *white arrow*) were observed in the case of all seven transgenic lines (lanes *3* through lane *9*, representing lines FM1, FM2, FM3, FM4, FM5, FM7, and FM8, respectively), but not in the case of wild-type plants (lanes *1* and *2*). Plasmid DNA (pW35SfMnP) was used as positive control (lane *10*). *M*, DNA markers (pHY Markers; Takara Bio, Otsu, Japan)

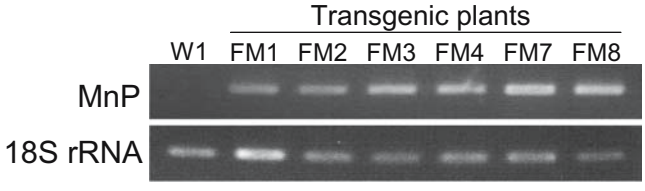

Fig. 2. Analysis of expression of the gene for manganese-dependent peroxidase (MnP) in transgenic lines of hybrid aspen. Reverse transcription PCR (RT-PCR) was performed with total RNA isolated from roots of the transgenic (*FM1* through *FM8*) and wild-type (*W1*) plants. 18S rRNA was subjected to RT-PCR as a control

we extracted total DNA from each plant and analyzed it by PCR. After PCR, each reaction mixture was fractionated on an agarose gel to confirm amplification of the target DNA. Figure 1 shows that amplified DNA of approximately 650 bp in length was detected only in the case of MnP-transgenic plants, and not in wild-type plants. All seven transgenic plants carried the transgene for MnP.

In order to confirm the expression of MnP in our transgenic lines, we also characterized the transgenic lines by RT-PCR, using total RNA isolated from each line. We used the same set of primers as those used for the analysis by PCR. We detected a specific amplified fragment in the analysis of six transgenic lines but not in wild-type plants (Fig. 2). Our data indicated that the introduced MnP-encoding cDNA was stably expressed in each transgenic plant, as observed previously in transgenic tobacco plants.[12] Under our experimental conditions, the expression of MnP gene led to no phenotypical differences between the MnP-expressing and wild-type plants (data not shown).

The introduced cDNA encoded a mature fungal MnP with a predicted secretion signal at its amino terminus end. WoLF PSORT analysis (http://wolfpsort.seq.cbrc.jp/) predicts that the amino-terminal sequence of MnP contributes to the extracellular sorting of the protein in both fungi and plants. In a previous study, we detected MnP activity in the hydroponic medium in which we had cultured MnP-expressing tobacco plants.[12] Successful production and subsequent secretion of a fungal laccase from roots of transgenic

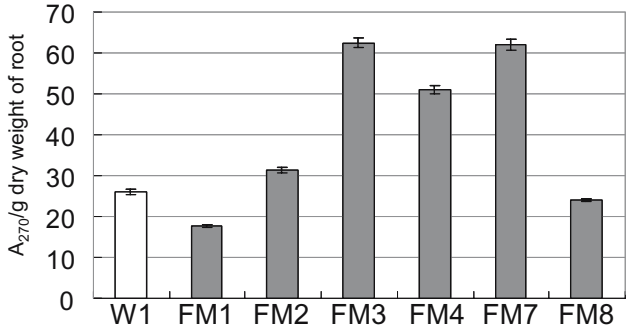

Fig. 3. The activity of MnP in the hydroponic medium, measured after incubation of undamaged root tissues from the wild-type (*W1*) and six transgenic plants. *Error bars* show standard deviation

tobacco plants was also detected in tobacco transformed with a full-length gene for laccase from *T. versicolor*.[14] Thus, fungal secretion signals appear to function in secretion of fungal proteins from transgenic plants. As in our previous studies, we determined the activity of MnP that had been secreted into the hydroponic medium used for the culture of our transgenic plants. Figure 3 shows the activity of secreted MnP in the culture medium, expressed as absorbance at 270 nm, which was due to the formation of manganese(III) malonate. Although the activity varied among the transgenic lines, the activities of plants in three lines, FM3, FM4, and FM7, was apparently higher than that of wild-type plants. Although the activities of the three lines were more than twice that of wild-type plants, the activity was low compared with those of MnP-transgenic tobacco plants found in our previous studies. The activity of the MnP-transgenic tobacco plants with the highest activity among the tested lines was approximately 54 times higher than that of wild-type plants.[12] The difference between transgenic tobacco and transgenic hybrid aspen might have been due to differences between species, the efficiency of expression of the transgene in each species, and/or to the number of lines analyzed (29 lines of transgenic tobacco and only 6 lines of transgenic hybrid aspen).

To test the activity of the transgenic hybrid aspen against BPA, we transferred four MnP-expressing transgenic plants to liquid medium that contained 100 μM BPA. Figure 4 shows that MnP-producing transgenic plants contributed to the disappearance of BPA from the medium, as also observed in the case of transgenic tobacco plants that produced a fungal laccase.[15] Although the reduction in level of BPA was not directly correlated with the activity of MnP secreted by each transgenic plant, the BPA-removing activities of all the MnP-expressing plants were more than twice that of the wild-type line. Thus, the secreted MnP protein from the transgenic plants may contribute, in part at least, to reduce BPA level in the medium. However, this result also suggests that the BPA-removing activity in the transgenic plants was not only due to the effect of MnP protein present in the medium. As indicated in previous reports,[5,6] BPA can be incorporated into plant cells and then metabolized to other compounds. In fact, apparent reduction in BPA level was also observed in the control line (Fig. 4). It

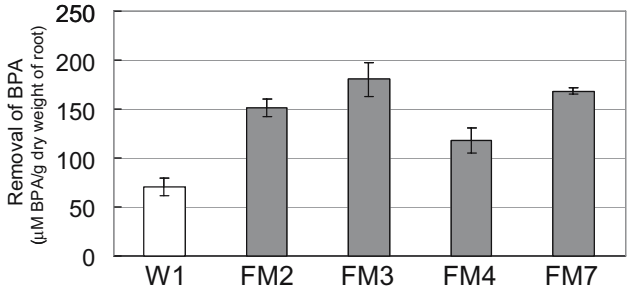

Fig. 4. Removal of bisphenol A (BPA) from the medium by MnP-producing transgenic plants. After hydroponic culture in the presence of BPA, as described in the text, levels of BPA were analyzed by high-performance liquid chromatography. The absorbance of the eluate was monitored at 278 nm. The values shown are the averages of results of two independent experiments with each line, with standard deviation shown by *error bars*

may be reasonable to think that MnP activity in the transgenic cells, not in the medium, can also contribute to the reduction of BPA level. In any case, future studies will require in-depth analysis to assess the direct contribution of MnP derived from the transgene to the removal of BPA.

This is the first report, to our knowledge, of a transgenic woody plant that secretes recombinant MnP from its roots into the rhizosphere. Production of the recombinant protein in roots offers new options for the use of woody plants in phytoremediation of soil and water, and, for example, for the efficient production of fungal proteins in plants.[16,17]

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