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Mercury-tolerant Transgenic Poplars Expressing Two Bacterial Mercury-metabolizing Genes

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Mercury is one of the most toxic metals to various organisms, including humans. Genes involved in mercury metabolism have been cloned from *Staphylococcus aureus*, and were modified here to be expressed in plants. Transgenic poplars containing both chimeric genes (p35S-*merA* and p35S-*merB*) were developed via two rounds of transformation using *nos-nptII* and *nos-hpt* genes as selectable markers. Although expression levels varied among transgenic lines, tolerance to either ionic mercury or organic mercury matched well with the degree of expression revealed by northern hybridization. In culture, these trees were tolerant to 50 μM $HgCl_2$ and 2 μM CH_3HgCl. Variations in mercury tolerance among the transgenic lines indicates that vigorous selection is required to select the best clones for use in phytoremediation.

Key words: *merA*, *merB*, mercury, phytoremediation, poplar, transformation

Mercury is one of the most toxic heavy metals. Soils polluted with Hg pose a severe challenge for both the health and the inhabitants of an ecosystem. The conventional technology for reducing mercury contamination involves the removal and off-site disposal of all soils at approved facilities. However, this is impractical and expensive to apply over large polluted areas. Recently, a phytoremediation approach has been proposed, based on the fact that plants generally absorb large amounts of essential elements and, in the process, they also take up toxic substances (Chaney et al., 1995; Salt et al., 1995; Raskin et al., 1997). Thus, compared with conventional physical reclamation methods, this approach is cost-effective and less disruptive because no disposal sites are needed (Krämer and Chardonnens, 2001). However, a number of problems must be resolved before using plants for large-scale soil remediation: 1) the roots can remove substances from only the top 2 m of the soil, 2) it takes years to clean a site, and 3) efforts may not succeed where the level of contaminants is too high to support plant growth.

Nevertheless, at least two facts favor implementing phytoremediation techniques. First, mercury contamination is confined to the top 40 cm of soil; second, the concentration of total Hg typically ranges from 0.5 to 3000.0 μg Hg g^{-1} of soil (Revis and Osborne, 1989). Therefore, organisms with enhanced tolerance to mercury toxicity may provide an alternative solution. Although no plant species are yet known to have natural tolerance to mercury, resistance is widely reported among gram-negative and -positive bacteria (Laddaga et al., 1987). In the environment, organic mercury ($R\text{-}CH_2\text{-}Hg^+$) is degraded to volatile mercury (Hg^0) in two steps -- cleavage into $R\text{-}CH_3$ and Hg(II), then conversion of the latter to Hg(0) and H^+ at the expense of NADH. Two enzymes are involved in this process. Whereas the first step is mediated by lyase coded by the *merB* gene, the second is by mercury reductase coded by *merA* (Peters et al., 1991). Rugh et al. (1998) have developed transgenic yellow poplar (*Liriodendron tulipifera*) for mercury phytoremediation by transferring a bacterial *merA* gene, and have demonstrated that the transgenic plants grow vigorously in media containing naturally toxic levels of ionic mercury. Bizily et al. (1999) have cloned a microbial *merB* gene and expressed it in *Arabidopsis thaliana*, making those transgenic plants tolerant to a wide range of concentrations of monomethylmercuric chloride. Moreover, transgenic eastern cottonwood carrying both *merA* and *merB* show enhanced capability to process organomercury (Satu et al., 2007). We previously reported that a *merB* gene originated from *Staphylococcus aureus* could be used as an excellent plant selectable marker for genetic transformation (Choi et al., 2005).

In the present study, we expressed both *merA* and *merB* in *Populus* via two rounds of *Agrobacterium*-mediated transformation, and investigated their expression levels to determine the tolerance of double-transgenic phenotypes to both organic and inorganic mercury.

MATERIALS AND METHODS

Construction of Plant Expression Vectors

Both the *merA* and *merB* genes were PCR-amplified from a *Staphylococcus aureus* strain (KCTC 1916) obtained from the Korean Collection Type Culture at the Biological Resource Center in Korea. Primers flanking *merA* and *merB* were synthesized based on the sequence of Laddaga et al. (1987), as follows: MA1, 5'-ggg atc caa atg act caa aat tca tat-3'; MA2, 5'-gga gct cct gca aca tag cat tag at-3'; MB1, 5'-gga tcc caa atg aaa aat att tca gaa ttc tca-3'; and MB2, 5'-cga gct cga ttg acg cag ggctaa ttg cct a-3'. The Kozac consensus sequence ((t/c)ccaa) was added to the MA1 and MB1 primers because the genes were of bacterial origin. PCR started with 2 min at 95°C, then continued with 35 cycles of 94°C (20 s), 55°C (40 s), and 72°C (2 min). Whereas *merA* was cloned into

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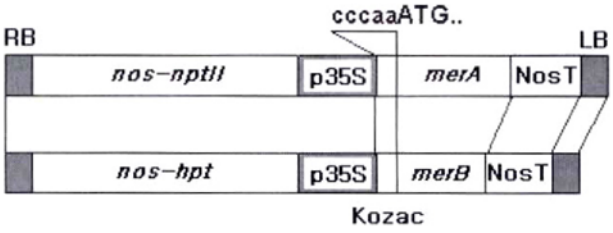

Figure 1. Schematic illustration of T-DNA regions containing chimeric *merA* (top) and *merB* (bottom) genes. *Nos-nptII*, neomycin phosphotransferase II gene under control of nopaline synthase promoter; *Nos-hpt*, hygromycin phosphotransferase gene under control of nopaline synthase promoter; p35S, 35S promoter of cauliflower mosaic virus; *merA*, mercury reductase coding sequence; *merB*, organomercurial lyase coding sequence; *NosT*, terminator sequence of nopaline synthase gene; RB, right border of T-DNA; LB, left border of T-DNA; Kozac, Kozac consensus sequence (cccaa) for eukaryotic (plant) expression.

binary vector pBI121 (Jefferson et al., 1987), *merB* was inserted into pBIB-HYG (Becker, 1990) by replacing the GUS gene at the *BamH1* and *Sac1* sites (Fig. 1).

Double Round of Plant Transformation and Regeneration

A non-flowering mutant hybrid poplar (*Populus alba* x *P. tremula* var. *glandulosa*) clone, BH1, was used for transformation with an *Agrobacterium tumefaciens* vector carrying p35S-*merA*. Protocols were as described by Choi et al. (2005). The transformed cells were selected on an MS medium containing 1.0 mg L^{-1} 2,4-D, 0.1 mg L^{-1} BAP, and 0.01 mg L^{-1} NAA (Murashige and Skoog, 1962), plus 500 mg L^{-1} cefotaxime and 50 mg L^{-1} kanamycin. Shoots were regenerated from the calli by transferring them to WPM (woody plant medium; Lloyd and McCown, 1981) containing 1.0 mg L^{-1} zeatin, 0.1 mg L^{-1} BA, and 0.01 mg L^{-1} NAA. The regenerated plants were used as materials for second-round transformation by p35S-*merB* with a selectable marker *nos-hpt* in the presence of 5 mg L^{-1} hygromycin. Throughout these experiments, the cultures were maintained at 25±2°C under a 16-h photoperiod provided from cool white fluorescent lamps (30 mole m^{-2} s^{-1}). The regenerated plantlets were then acclimated in a walk-in growth chamber for 2 months before being transferred to the greenhouse. After 2 months, the plants were moved to a nursery where they have since been maintained.

Southern and Northern Analyses

Genomic DNA was extracted from the leaves of nursery-grown poplar plants by using a MagExtractor-Plant Genome kit (Toyobo, Japan). Ten µg was digested overnight with restriction enzyme *Pst*I. The DNA was then run on a 1% agarose gel and transferred to a Hybond-XL nylon membrane by the capillary transfer method (Southern, 1975). Afterward, it was hybridized for 12 h with the ^{32}P-dCTP-labelled full-length *merA* or *merB* coding sequence. The membrane was washed in 2× SSC and 0.1% SDS (50°C) for 10 min and in 0.2× SSC and 0.1% SDS (50°C) for 30 min, followed by exposure to X-ray film at –70°C.

Steady-state mRNA levels of both p35S-*merA* and p35S-*merB* in the transgenic trees were also compared by northern blot analysis. Total RNA was isolated with TRI Reagent (Molecular Research Center, USA) and 10 µg was run on a 1.2% formaldehyde agarose gel before being transferred to a Hybond-XL nylon membrane. Pre-hybridization was conducted for 30 min at 68°C in a 1×PerfectHYB plus hybridization buffer (Sigma, USA) and 0.1 mg mL^{-1} denatured salmon sperm DNA solution. The remaining steps and probes were the same as for our Southern hybridization.

Assays of Tolerance to Mercury

Eight-week-old transgenic poplar plants carrying the p35S-*merA* and/or p35S-*merB* genes were used to determine their mercury tolerance. From each line, 30 leaf segments (0.5 cm × 0.5 cm) were prepared, and 10 each were placed on 90 mm × 15 mm Petri dishes containing 10 mL of a callus induction medium (Murashige and Skoog, 1962) with 1.0 mg L^{-1} 2,4-D, 0.1 mg L^{-1} BAP, and 500 mg L^{-1} cefotaxime, plus 0, 50, or 100 µM $HgCl_2$ and 2 or 5 µM CH_3HgCl. Increments in calli fresh weights were compared after 4 weeks of culture. Throughout these experiments, the cultures were maintained at 25±2°C under a 16-h photoperiod provided from cool white fluorescent lamps (30 mole m^{-2} s^{-1}).

Mercury Vapor Assay

The double-transgenic poplars were grown in an artificial soil mixture (1:1 vermiculite:perlite) containing 50 µM $HgCl_2$. These were placed in closed bottles and cultured for 4 d. Gaseous mercury was removed with a syringe and measured by a mercury vapor analyzer (Arizona Instrument, Tempe, USA) according to the manufacturer's recommendations. Mercury levels from plants grown in soil containing 50 µM $HgCl_2$ were also determined by an NIC SP-3B automatic mercury analyzer (NIC, Tokyo, Japan).

RESULTS AND DISCUSSION

Regeneration of Transgenic Plants

In our first round of transformation, we obtained about 30 transgenic plants containing the p35-*merA* gene. Regenerated shoots were transferred to a root induction medium (MS + 0.2 mg L^{-1} IBA) containing 50 mg L^{-1} kanamycin. The transgenic plants were then further screened by leaf-disc culture on a callus induction medium containing 50 mg L^{-1} kanamycin. After PCR-amplification with *merA* gene-specific primers, three *merA* transgenic plants were chosen for second-round transformation with the p35S-*merB* gene. Selection of these transformants was done in the presence of 5 mg L^{-1} hygromycin, and 12 lines were obtained. The regenerated double transformants were morphologically identical to the wild-type (WT) plants and grew normally under our culturing conditions. Southern blot analyses using both *merA* and *merB* coding sequences as probes confirmed that the double-transgenic poplars contained both genes as single copies. However, for two exceptions (Fig. 2), some of the regenerated plants did not contain the transgene(s): 1) *merB* was missing in Line 5 and 2) both genes were absent in Line 4.

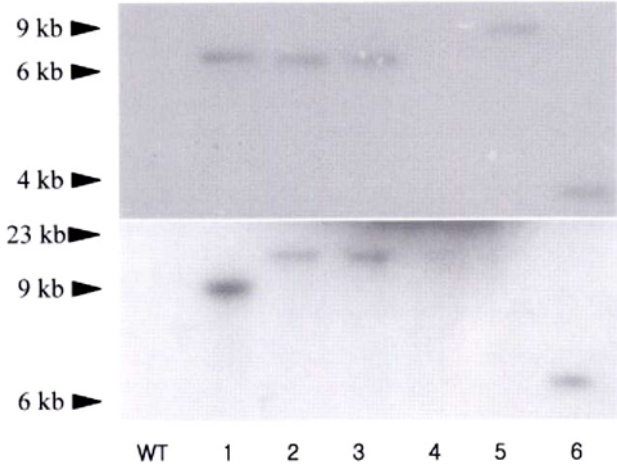

Figure 2. Southern blot analysis of double-transgenic poplars with *merA* (top) and *merB* (bottom) genes as probes. Both genes are missing in regenerant Line 4; *merB* is absent in Line 5.

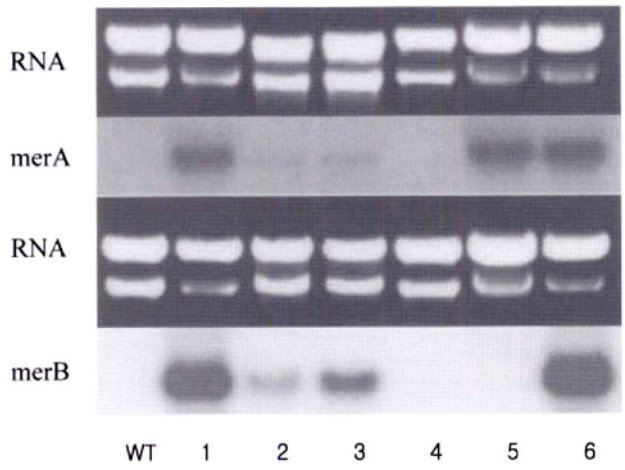

Figure 3. Northern blot analyses of *merA* (top) and *merB* (bottom) genes in double-transgenic poplars. Although both *merA* and *merB* transcripts were detected in all but two transgenic lines, their expression levels varied.

Expression of the Chimeric p35S-*merA* and p35S-*merB* Genes

Expression of *merA* and *merB* varied among the transgenic lines (Fig. 3). Overall, transcript levels of the p35S-*merA* gene appeared to be lower than those of p35S-*merB*. As expected, transgenic Line 4, which showed no *merA* signal in our Southern hybridization, did not express the gene. Likewise, Line 5 showed no signal or expression for either transgene. Among the transgenic lines, levels of expression varied by over 10-fold. Therefore, for further analyses, we chose two highly expressed transgenic lines (1 and 6) and one with mild expression (Line 3) to examine the correlation between mRNA expression and the extent of their tolerance to mercury.

Mercury Tolerance of Transgenic Plants

Tolerance to ionic mercury

Transgenic p35S-*merA* poplars grew slightly better than the wild type in the presence of 50 μM $HgCl_2$, but callus development varied among the p35S-*merA* transgenic lines. For example, whereas Line 6, with high *merA* activity (per the northern analysis), performed significantly better than the others, growth of transgenic Line 2, with a weak northern signal, was retarded to the level measured from the WT. However, the tolerance conferred by that transgene did not appear to be dramatic, in that the WT plants also survived when exposed to the same amount of ionic mercury. Nevertheless, while the *merA* transgenic calli remained yellow-green, those from the nontransgenics turned brown during the 4-week culture period. Similar observations have been made with peanut plants, where treatment with 50 μM ionic mercury still allows some embryos to be produced from their embryogenic mass (Yang et al., 2003). However, our results contrast with those of Che et al. (2003), who have shown that all non-transgenic cottonwood (*Populus deltoides*) lines are killed at 25 μM $HgCl_2$. In our present study, none of the transgenic lines succumbed at 100 μM $HgCl_2$.

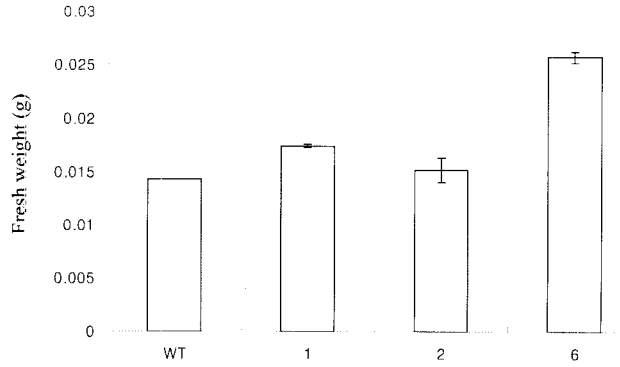

Figure 4. Fresh weight growth of calli from p35S-*merA* transgenic poplar lines in presence of 50 μM $HgCl_2$. Culture period was 4 weeks.

The data presented in Figures 5 and 6 provide reasons for using transgenic p35S-*merA* poplars to remove inorganic mercury from polluted soil. We conducted mercury vapor assays to analyze the reduction of toxic $HgCl_2$ to much less harmful Hg^0. Here, transgenic Line 6, which showed high

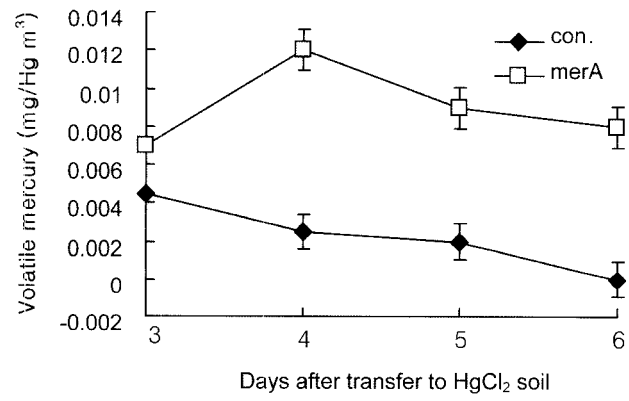

Figure 5. Volatile Hg^0 generated by p35S-*merA* transgenic plants in closed culture bottle. Reduction of $HgCl_2$ to Hg^0 was monitored by mercury vapor analyzer.

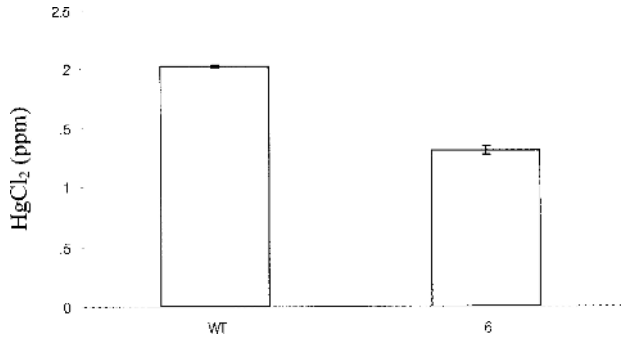

Figure 6. *In planta* Hg contents per individual p35S-*merA* transgenic plants.

merA expression in the northern analysis, produced 4.5~4.8 times more Hg^0 than did the control plants when grown in an artificial soil mixture containing 50 μM $HgCl_2$. Mercury content also was lower in the transgenics than in the untransformed control (Fig. 6). Taken together, we suggest that the transgene p35S-*merA* confers to transformed poplars a limited tolerance to ionic mercury but also enables its more efficient removal compared with WT plants.

Tolerance to organic mercury

Three double-transgenic lines (two with high expression, Lines 1 and 6; and one with mild expression, Line 3) were tested for their tolerance to organic mercury (2 or 5 μM CH_3HgCl). The two highly expressing lines grew significantly better than the non-transgenic plants (Fig. 7). Although the mildly expressing line survived and remained healthy when exposed to 2 μM CH_3HgCl, it did not grow well, suggesting it had only limited tolerance to methylmercury. Tolerance of the transgenic lines was closely associated with levels of northern expression. However, none of those transformants survived treatment at 5 μM methylmercury.

All the above results consistently demonstrate that heterologous expression of the chimeric p35S-*merA* and p35S-*merB* genes confers tolerance to both inorganic and organic mercury by transgenic poplar. Moreover the degree of tolerance in each line is correlated with transgene expression levels, suggesting that the best transgenic clones must be selected for use in field applications. Between the two, *merB* appears to have a more dramatic effect, possibly because of its easy penetration and translocation across the membrane. In contrast, ionic mercury could interfere with membrane function and, thus, not be transported to the cytoplasm where degradation occurs.

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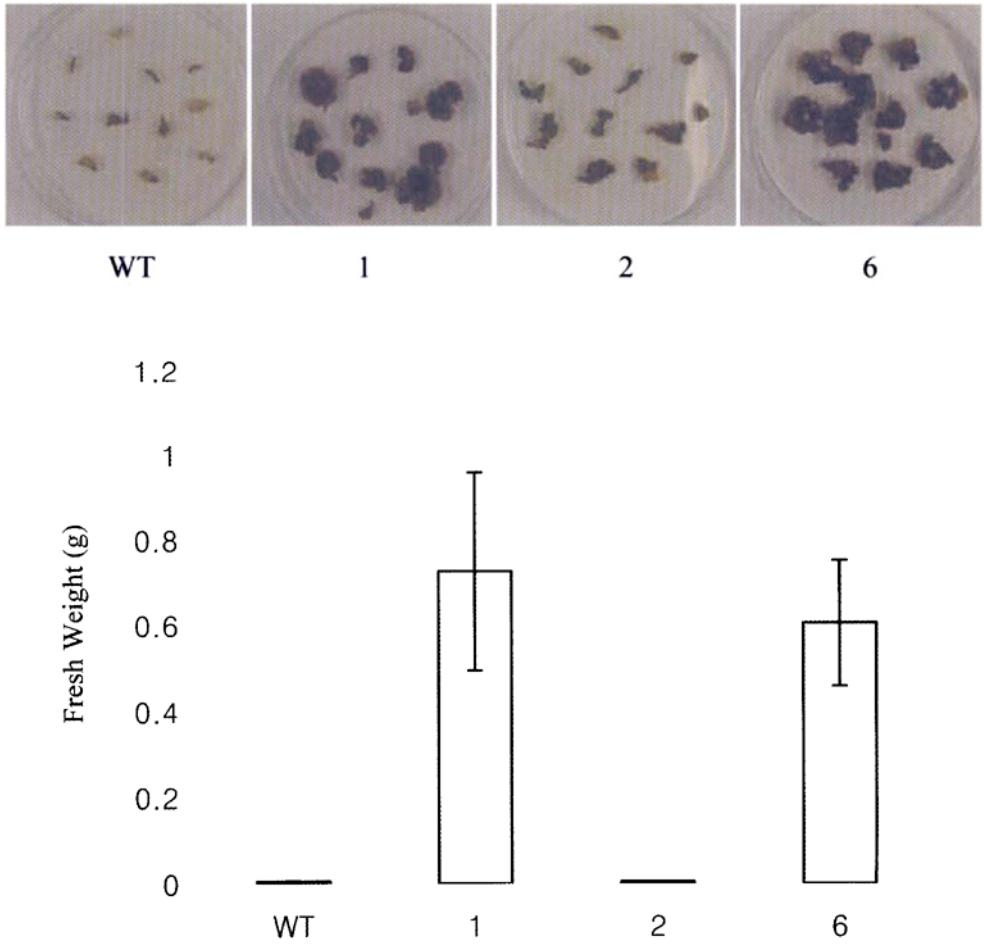

Figure 7. Fresh weight growth of calli from *merB* transgenic poplar lines in presence of 2 μM CH_3HgCl.

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