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# An Efficient and Novel Plant Selectable Marker Based on Organomercurial Resistance

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**A selectable marker gene facilitates the detection of genetically modified plant cells during transformation experiments. So far, these marker genes are almost exclusively of two types, conferring either antibiotic resistance or herbicide tolerance. However, more selectable markers must be developed as additional transgenic traits continue to be incorporated into transgenic plants. Here, we used mercury resistance, conferred by the organomercurial lyase gene, as a selectable marker for transformation. The *mer*B gene from *Streptococcus aureus* was modified for plant expression and transferred to a hybrid poplar (*Populus alba* x *Populus glandulosa*), using the stem segment-agrobacteria cocultivation method. The transformed cells were selected on a callus-inducing medium containing as little as 1 μM methylmercury. Subsequent plant regeneration was done in the presence of methylmercury. Resistance to Hg was stably maintained in mature plants after two years of growth in the nursery. We suggest that this gene could serve as an excellent selectable marker for plant transformation.**

*Keywords:* merB, mercury, organomercurial lyase, plant transformation, selectable marker

The introduction of genes into plants requires selectable markers. The most commonly used in transformations is those for antibiotic resistance because of their high efficiency and reproducibility. These markers include resistance to kanamycin (Fraley et al., 1983), hygromycin (Waldom et al., 1985), streptomycin (Jones et al., 1987), gentamycin (Hayford et al., 1988), phleomycin (Perez et al., 1989), or streptothricin (Jeleska et al., 2000). Recently, novel markers based on substrate-dependent negative selection have also been reported (Babwah and Waddell, 2000). With the increasing number of genes now available, more selectable markers must be developed to transfer new traits into already transformed plants.

Mercury resistance, as conferred by the organomercurial lyase gene, is known among gram-negative and -positive bacteria (Laddaga et al., 1987; Peters et al., 1991). Both *merA* and *merB* genes from *Escherichia coli* are expressed in transgenic *Arabidopsis thaliana* (Bizily et al., 1999). Those plants carrying both *merA* (for resistance to mercury chloride) and *merB* survive in the presence of up to 5 μM methylmercury ($CH_3HgCl$), converting that element to a much less toxic volatile form ($Hg^0$). Although the *merA* gene is also utilized as a selectable marker for plant transformation (Yang et al., 2003), the *merB* gene had not been tested yet as a potential marker. Here, we examined whether the *merB* gene from *Staphylococcus aureus* could be used as a novel selectable marker for transformation of a *Populus* hybrid.

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## MATERIALS AND METHODS

### Vector Construction

The gene for organomercurial lyase (*merB*) was PCR-amplified from plasmid DNA extracted from *S. aureus*, based on the sequence reported by Laddaga et al. (1987). Primers for this amplification comprised two oligonucleotides, P1: 5′-CGGATCCCAAATGAAAAATATTTCAGAATTCTCA-3′ and P2: 5′-CGAGCTCGATTGACGCAGGGCTAATTGCCTA-3′. For plant expression of the gene, a ribosomal binding site (CCCAA) was included in the P1 primer. To aid in efficient cloning, the *Bam*HI site was added to the P1 primer, and the *Sac*I (or *Sst*I) site to the P2 primers. A T-DNA vector was constructed by replacing the GUS gene from binary vector pBI121 with the modified *merB* gene at the *Bam*HI and *Sac*I sites (Jefferson et al., 1987). Thus, the T-DNA construct pMerB contained the *merB* gene, placed between the 35S pro-

moter of cauliflower mosaic virus (CaMV), and the terminator of the nopaline synthase gene of *Agrobacterium tumefaciens* plus the *nos-nptII* gene. This recombinant plasmid was then transferred to *A. tumefaciens* strain LBA4404 by the freeze and thaw method (An et al., 1988).

### Transformation and Regeneration of Transgenic Plants

Plant transformation was done by co-cultivating the stem segments of a hybrid poplar (*Populus alba x P. glandulosa*) with *A. tumefaciens* carrying the recombinant plasmids. The *Agrobacterium* was grown at 30°C overnight in 50 ml YEP [0.5% (w/v) beef extract, 0.1% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) sucrose, and 2 mM $MgSO_4$]. The bacteria were then pelleted by centrifugation at 1080*g* for 10 min at 4°C, and re-suspended in 20 mL of an 0.85% NaCl solution. After adding acetosyringone (3′,5′-D-methoxy-4′-hydroxy-acetopeptone) (final concentration of 100 μM) to the *Agrobacterium* suspension, poplar stems growing in a test tube were cut into small pieces (ca. 5 mm long) and soaked in the suspension for 20 min. They were then transferred to a 50-mL conical tube containing 20 mL of 0.85% NaCl solution. The tube was gently shaken to release excess bacteria. Afterward, the stem segments were gently blotted between two sterile paper towels and transferred to a solidified MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg $L^{-1}$ BA and 1.0 mg $L^{-1}$ 2,4-D. Two days later, the explants were placed on a medium containing 5 M methylmercury. After 2 to 3 weeks, calli were visible from the cut edges of the stem segments. Shoots regenerated from the calli were transferred onto a WPM (Lloyd and McCown, 1981) containing 1.0 mg $L^{-1}$ zeatin, 0.1 mg $L^{-1}$ BA, 0.01 mg $L^{-1}$ NAA, and 5 μM $CH_3HgCl$. The regenerated shoots were micropropagated via shoot tip culture and placed on a rooting medium (MS + 0.2 mg $L^{-1}$ IBA + 5 μM $CH_3HgCl$). Throughout the experiments, transformed cells were selected only by 5 μM methylmercury. The plants were assayed first by culturing on a medium contai- ning kanamycin (50 mg $L^{-1}$); second, by growing them on both solidified and liquid media containing 5 μM methylmercury; and third, via RT-PCR with *merB* primers.

### Genomic DNA Isolation and Southern Blot Analysis

Genomic DNA was extracted with a MagExtractor-Plant Genome Kit (Toyobo, Japan) from the leaves of poplar plants growing in test tubes or the nursery. Ten μg of genomic DNA was digested overnight with the restriction enzyme *PstI*. The DNA was then run on a 1% (w/v) agarose gel and transferred to a Hybond-XL nylon membrane by the capillary transfer method (Southern, 1975). It was then hybridized for 12 h with the $^{32}$P-dCTP-labeled full-length *MerB* coding sequence. Afterward, 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.

### Northern Blot Analysis

Total RNA was isolated from young leaves using TRI Reagent (Molecular Research Center, USA). Ten μg was run on a 1.2% formaldehyde agarose gel and transferred to a Hybond-XL nylon membrane. The membrane was pre-hybridized for 30 min at 68°C in 1× PerfectHYB plus hybridization buffer (Sigma, USA) and 0.1 mg $mL^{-1}$ denatured salmon sperm DNA solution. It was then hybridized for 12 h with the $^{32}$P-dCTP-labeled full-length *tzs* coding sequence. The membrane was next 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.

Reverse transcription (RT) PCR was performed using 1 μg of RNA with the primers merB1 and merB2, as described by Wang et al. (1992).

## RESULTS AND DISCUSSION

### Effect of Methylmercury on Selection of Transformed Cells

When the co-cultivated stem segments were grown at a low concentration (e.g., 0.2 μM) of methylmercury, agrobacteria proliferated on the media. However, no overgrowth was detected at a level of 2 μM (or higher) during the culture period, even in the absence of cefotaxime. The frequency of callus formation on the medium containing 5 μM methylmercury ranged from 70 to 80%, depending on the explant material (data not shown). In the early stage of induction, the calli were yellow but gradually changed to red. However, at a concentration of at least 2 μM, no calli were induced from untransformed stem segments used as our control.

Shoots were regenerated from the calli upon their transfer to an induction medium containing 5 μM

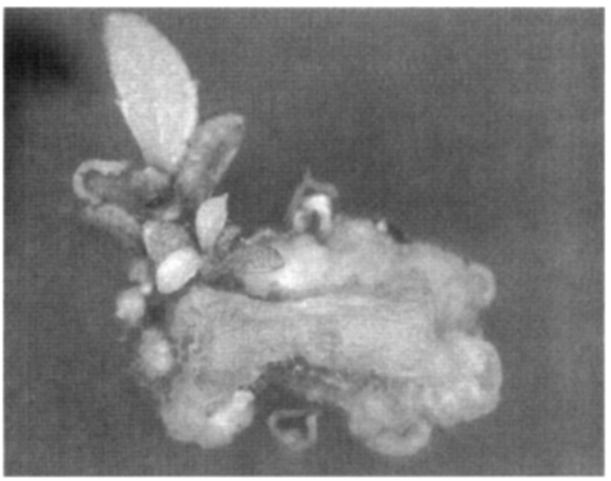

**Figure 1.** Selection and regeneration of poplar plants after *Agrobacterium*-mediated transformation in the presence of 5 μM methylmercury.

methylmercury (Fig. 1). We routinely transform poplar with vectors carrying either *nos-nptII* or *nos-hpt*. There appeared to be little difference in transformation efficiency between the use of *merB* and either *nptII* or *hpt*.

For shoot regeneration, about 50 calli were transferred to an induction medium containing 5 μM methylmercury; almost all produced shoots. These plantlets then formed roots on a medium containing 5 μM methylmercury (data not shown). Neither the untransformed plants nor the control calli survived on either callus-induction media or plant-regeneration media containing 5 μM methylmercury (Fig. 2).

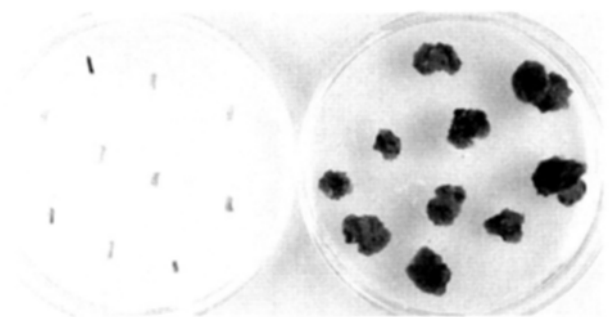

**Figure 2.** Confirmation of transformation by growing stem tissues from transgenic poplar in presence of methylmercury. Excised stem segments were cultured for 4 weeks on callus-inducing medium containing 5 μM methylmercury. Whereas explants from untransformed control plants formed no calli and died on medium (left), those of transformed plants formed calli in the presence of methylmercury (right).

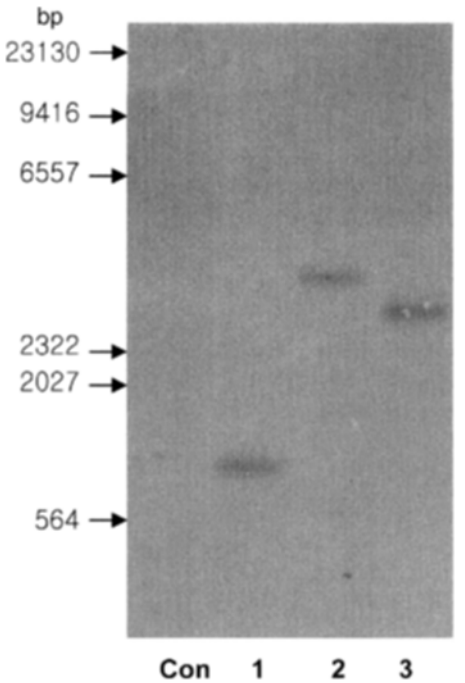


**Figure 3.** Southern blot analysis of poplar plants transformed with 35S-merB gene and selected in the presence of methylmercury. Genomic DNA (10 μg) from regenerated plants was digested with *Pst*I and separated by electrophoresis in 1.0% agarose gel. The gel was blotted onto nylon membrane, and hybridized with $^{32}$P-labeled full-length *merB* gene. Numbers on left indicate size of DNA markers in bases. Con, control plant; 1,2, and 3, transformed plants.

## Southern and Northern Analyses

Our Southern analysis of transformed plants, using the *merB* coding sequence as a probe, confirmed the presence of a single-copy transgene (Fig. 3). The northern analysis showed that this transgene was well expressed in the transgenic plants (Fig. 4).

## Effect of Methylmercury on Growth of Transgenic Plants Carrying the *merB* Gene

Figure 5 presents the curves typically obtained from non-transformed control plants (WT) and *merB* transgenic plants grown on callus-induction media in the presence of varying concentrations of methylmercury. At the level of 2 μM, no calli were induced from the untransformed tissues. However, most transgenic plants were not affected even at a concentration of 5 μM. Overall, 2 μM methylmercury was sufficient to select transformed cells (Fig. 5). Because Hg is not an essential plant element, it is impossible to know the exact mechanism by which both transformed and

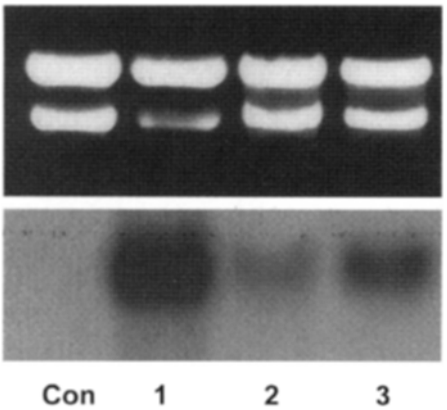


**Figure 4.** Expression of 35S-merB gene revealed by northern blot analysis. Ten μg of total RNA extracted from leaf tissues was resolved on formaldehyde agarose gel and blotted onto nylon membrane. Hybridization was done with $^{32}$P-labeled probe of full-length *merB* gene. At top is EtBr-stained gel showing rRNA bands. Con, control plant; 1, 2, and 3, transgenic plants.

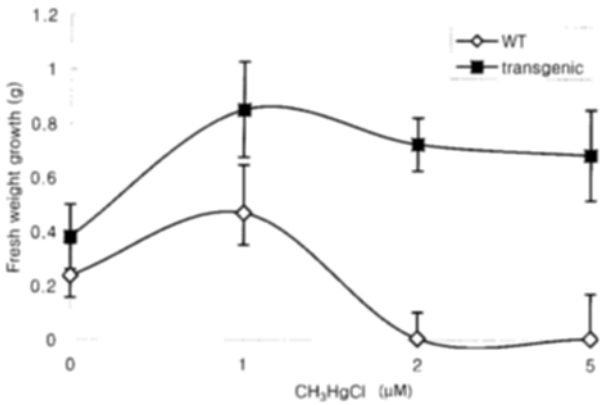


**Figure 5.** Dose-response curve for growth of poplar cells in the presence of methylmercury. Calli were formed from stem explants excised from transgenic plants carrying chimeric 35S-merB gene. Fresh weights of calli were determined after 4 weeks of culture on MS + 0.1 mg $L^{-1}$ BA and 1.0 mg $L^{-1}$ 2,4-D in the presence of various concentrations of methylmercury.

untransformed cells grew better in the presence of 1 μM methylmercury than in its absence. Similar results have been observed in poplar cell cultures treated with paraquat (Choi et al., 2001) and in the naked barley cultivar treated with $AlCl_3$ (Jeong and Kim, 2004).

Our results demonstrate that the organomercurial lyase gene, *merB*, could be an excellent plant selectable marker. However, one drawback to its adoption may be the toxicity of methylmercury used for the selection. Utilizing both *merA* and *merB* could be helpful because those genes work together, in a two-step process, to convert methylmercury to less toxic mercuric chloride and then to volatile mercury ($Hg^0$). Nevertheless, the *merB* gene could still serve as an alternative selectable marker for multiple gene-transfer experiments where other markers are no longer available.

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