Engineering Forest Trees with Heavy-Metal Resistance Genes for Phytoremediation

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Over the past century, mining, agriculture, manufacturing and urban activities have all contributed to extensive contamination of soil and water with heavy metals. In the United States, mercury is a common pollutant at government production sites, where it is used in energy- and defense-related activities. Thousands of square miles of land, rivers, lakes and estuaries are contaminated with millions of kilograms of mercury. Methylmercury, produced by bacteria in contaminated aquatic areas, is particularly toxic. Because it is quickly biomagnified in the food chain, it can have devastating effects on humans and other animals. Another common pollutant in the United States and worldwide is arsenic, a naturally occurring element widely distributed in the earth's crust. In the environment, arsenic combines with oxygen, chlorine, and sulfur to form inorganic compounds. These extremely toxic metalloids, classified as "group A" human carcinogens, cause skin lesions, lung, kidney and liver cancer, and damage to the nervous system. Conventional procedures for cleaning up heavy-metal-contaminated sites (*i.e.* excavation, dredging, electrolytic extraction, chemical leaching) are all prohibitively expensive and destructive of the natural environment. An alternative to these physical remediation approaches is the use of plants to remove pollutants from soil and water through their root systems, an approach known as phytoremediation. Once extracted, plants may sequester the pollutants in their tissues and/or convert them to less toxic forms. They can accomplish this at a fraction of the cost most physical/chemical methods and without disrupting the environment. Although some plants, known as hyperaccumulators, can take up and sequester normally toxic amounts of heavy-metal pollutants, most of these species accumulate little biomass, and thus are probably not suitable for rapid remediation of extensive areas. An alternative approach is to genetically engineer plants possessing faster growth and greater biomass-accumulation potential with genes allowing them to handle these pollutants. Forest trees, in particular, with their large biomass and penetrating root systems, make excellent candidates for engineering with phytoremediation genes. Such an approach is under development at the University of Georgia, where a number of plant species, including some forest trees, have been engineered with genes from bacteria that have been modified to function efficiently in plants. Our work indicates that forest trees can be engineered to thrive on and detoxify a variety of heavy metals on polluted sites.

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Engineering Trees for Mercury Phytoremediation

Mercury-resistant bacteria express MerA to convert highly toxic mercuric ion, Hg(II), to much less toxic elemental mercury, Hg(0). Following a demonstration that a modified *merA* gene conferred mercuric ion resistance to *Arabidopsis* plants (Rugh *et al.,* 1996), we used embryogenic culture (Merkle *et al.,* 1990) and gene-transfer systems (Wilde *et al.,* 1992) that we had previously developed for the fast-growing forest species yellow-poplar (*Liriodendron tulipifera*) to generate trees expressing a modified *merA* gene (Rugh *et al.,* 1998). Yellow-poplar proembryogenic masses (PEMs) were transformed with three modified *merA* constructs via microprojectile bombardment. Each construct was synthesized to have altered flanking regions with stepwise increases (0%, 9%, and 18% blocks) of modified coding sequence. All of the *merA* constructs that we tested conferred resistance to toxic ionic mercury that had been incorporated into the tissue-culture medium. Yellow-poplar somatic seedlings containing the most modified *merA* gene (*merA18*) germinated and grew vigorously in media containing a normally toxic level (50 μ*M*) of ionic mercury. A mercury volatilization assay indicated that the *merA18* plantlets released elemental mercury at approximately ten times the rate of untransformed control plantlets, indicating that they efficiently reduced mercuric ion to the elemental form.

While our work with yellow-poplar demonstrated the potential to engineer a forest tree with mercury-handling genes, we did not test these trees outside of *in vitro* conditions. Yellow-poplar is not adapted to the wet soils or riparian sites where mercury contamination is a major problem. For this reason, we proceeded to look for another tree species to engineer with mercury-handling genes. *Populus*, a forest-tree genus of the Salicaceae that includes aspens and cottonwoods, is easy to establish and grows quickly. Its high transpiration rate and wide-spreading root system make it ideal to intercept, absorb, degrade and/or detoxify contaminants, while reducing soil erosion (Dix *et al.,* 1997). Many *Populus* species, in particular the cottonwoods, are especially adapted for growth on riparian sites, making them a good choice for establishment on sites requiring remediation. In addition, *Populus* is amenable to *in-vitro* propagation and genetic engineering (*e.g.* Han *et al.,* 2000), making it a suitable target for enhanced phytoremediation ability via transgenic technology. Non-transgenic poplars had already been tested by some groups for phytoremediation applications (Licht, 1990; Newman *et al.,* 1997; Burken and Schnoor, 1997). We used *Agrobacterium*-mediated transformation of leaf explants to generate transgenic eastern cottonwood (*P. deltoides*) trees expressing *merA9* and *merA18* genes. Leaf sections from transgenic plantlets produced adventitious shoots in the presence of 50 μ*M* Hg(II), supplied as $HgCl_2$, which completely inhibited shoot induction from leaf explants of wild-type plantlets. Transgenic shoots cultured in medium containing 25 μ*M*

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Hg(II) rooted and showed normal growth, whereas wild-type shoots were killed. When the transgenic cottonwood plantlets were exposed to mercuric ion, they evolved two to four times the amount of Hg(0) relative to wild-type plantlets. Transgenic *merA9* trees tested in a Georgia Piedmont soil contaminated with approximately 400 ppm Hg(II) showed growth indistinguishable from those in uncontaminated soil, while control plantlets were completely defoliated and dead within a week following potting (Che *et al.,* 2003).

Trees of one *merA* eastern cottonwood clone, along with wild-type control trees (approximately 200 trees total) were planted on a mercury-contaminated site that was formerly the location of a hat-making factory in Danbury, CT, in June 2003; *merA* and control trees all grew well. Leaves were sampled from *merA* and control trees in fall 2004, and analysis indicated that those from the *merA* trees contained one-third to one-half the amount of Hg as leaves from the control trees. This result was expected since the *merA* trees produce and volatilize Hg(0), which is then able to leave the plants as a vapor, whereas the control trees are unable to do so.

Methylmercury (MeHg), produced by native bacteria at mercury-contaminated wetland sites, is more toxic than elemental or ionic mercury. Because it is efficiently biomagnified up the food chain, it poses the most immediate threat to animal and human populations. Building on work performed in *Arabidopsis* (Bizily *et al.,* 1999), we produced eastern cottonwood shoots engineered with a bacterial *merB* gene, which converts MeHg to Hg(II). These shoots expressed the MerB protein and demonstrated their resistance to the methylmercury analog phenylmercuric acetate (PMA) by producing longer adventitious roots and higher fresh weights than control shoots cultured on rooting medium supplemented with 2 or 5 μ*M* PMA (Che *et al.*, submitted). However, in order to remove organic mercury from a contaminated site, it is desirable to have trees expressing both the *merA* and *merB* genes, so that organic mercury compounds can ultimately be converted to the least toxic form, elemental mercury. Results with *Arabidopsis* indicated that combining the *merA* gene with the *merB* gene in the same plant can increase the ability to grow on levels of organic mercury up to fifty-fold higher than wild-type plants and up to ten-fold higher than plants engineered with *merB* alone (Bizily *et al.,* 2000). This enhanced resistance to organic mercury is probably due to the fact that plants expressing the *merA* and *merB* genes together are able to transform both organic and ionic mercury to volatile Hg(0). Thus, the goal of our recent research has been to engineer both genes into eastern cottonwood. To accomplish this, we developed a system to re-transform *merA* cottonwood trees with the *merB* gene. Preliminary results comparing the *merA/B* trees to wild-type controls and trees engineered with either *merA* or *merB* alone indicate that these *merA/B* trees can efficiently convert PMA to Hg(0) (Lyyra *et al.*, in preparation).

We plan to engineer these same gene combinations into eastern cottonwood and other trees and test their potential for arsenic remediation.

Engineering Trees for Arsenic Phytoremediation

Using similar *in-vitro* culture and gene-transfer methods to those we employed with *merA* and *merB*, we engineered a bacterial γ-glutamyl synthetase (γECS) gene into eastern cottonwood. A month after being cultured on the medium supplemented with 800 μ*M* arsenate, leaf sections from γECS transgenic lines remained green and began to develop callus, while the leaf sections from wild-type plantlets showed no evidence of callus and became chlorotic. After 30 days on medium containing 800 μ*M* arsenate, wild-type adventitious shoots did not form roots and their leaves became chlorotic. The γECS shoots appeared similar to those maintained on medium with no arsenate, and adventitious roots began to appear at 21 days after initial culture. The difference between the γECS lines and the wild-type plants in their abilities to produce adventitious roots in medium with 800 μ*M* arsenate was statistically significant (Lima *et al.*, in preparation). Despite the apparent slight enhancement of arsenate resistance conferred to our eastern cottonwood trees by the γECS gene, work with transgenic γECS *Arabidopsis* plants indicated that they removed no more arsenate from the medium than did wild-type control plantlets (R.B. Meagher, unpublished data). Thus, not only does the mechanism of arsenate resistance for γECS plants remain unknown, but it is unlikely that engineering plants with this gene alone will be useful for removing arsenic from contaminated soil or water. Recent research in which the γECS gene was combined with other arsenic-handling genes, such as arsenate reductase, in transgenic *Arabidopsis* plants indicates that some of these multigene approaches have promise (Dhanker *et al.,* 2002). Thus, we plan to engineer these same gene combinations into eastern cottonwood and other trees and test their potential for arsenic remediation.

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