The Phytoremediation of Ionic and Methyl Mercury

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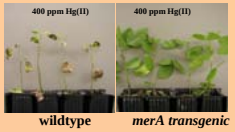


Wild-type and *merA* transgenic Eastern Cottonwood plantlets were grown on uncontaminated soil, transferred to mercury contaminated soil from Oak Ridge National Laboratory for 10 days, and photographed. Similar *merA* plants are in field trials.

Abstract. Hypothesis & Model

Our current working hypothesis is that transgenic plants controlling the transport, chemical speciation, electrochemical state, volatilization, and aboveground binding of mercury will: a) tolerate mercury and grow rapidly in toxic mercury contaminated environments; b) prevent methylmercury from entering the food chain; c) remove mercury from polluted sites; and d) hyperaccumulate mercury in aboveground tissues for later harvest. The molecular genetic strategies suggested by this hypothesis are dissected in **Figure 1** . Various parts of this hypothesis are being tested by examining different transgenes in model plants and comparing the results to control plants. By expressing some genes in roots, some in leaves, and some constitutively, we can further improve the efficiency of these processes. At present we are working with over 20 different transgenes and several different promoter expression systems as part of this project. In the next two years we will construct mercury processing super-plants combining the severak transgenes needed to for high level mercury hyperaccumulation aboveground (e.g., 1000 ppm). Background on previous DOE funded research (**Figures 1-3 & Bibliography**) and the preliminary results from the first 9 months of research our our latest project (**Figures 4-6**) are presented herein.

Background

Our long-term goal is to enable highly productive plant species to extract, resist, detoxify, and sequester the toxic heavy metal mercury. Mercury pollution, primarily due to methylmercury (MeHg, CH_3Hg^+) produced by native bacteria at mercury contaminated wetland sites, is a world-wide problem seriously affecting the health of human and wildlife populations. MeHg is inherently more toxic than metallic Hg(0) or ionic Hg(II) mercury, and because MeHg is efficiently biomagnified up the food chain, it poses the most immediate threat to animal populations.

As part of earlier DOE EMSP funded research, we successfully engineered several plant species to use the bacterial *merB* gene, methylmercury lyase, to convert MeHg to less toxic Hg(II) (**Figure 2**), and to use a highly modified bacterial mercuric ion reductase gene, *merA*, to further detoxify Hg(II) to the least toxic metallic form Hg(0), which is volatilized from plant tissues. Plants expressing both MerA and MerB proteins detoxify MeHg to Hg(0) in two steps. All these plants germinate, grow, and set seed at normal growth rates on levels of MeHg or Hg(II) that are lethal to wild-type plants, thus meeting one of the most important goals of phytoremediation: resistance to the toxic pollutant. During the last three year grant period, we achieved 90% of the milestones set under six Specific Aims including: 1) developing a strong, root-specific gene expression system (Kim *et al.*, 2005); 2) increasing the aboveground binding of mercury using low-molecular weight chelating agents (Li *et al.*, 2004; Li *et al.*, 2005; Zimeri *et al.*, 2005; Li *et al.*, 2006a); 3) combining multiple transgenes that improve mercury tolerance and uptake (Li *et al.*, 2006b); 4) developing a system of complete female-male-sterility to assist in the containment of genetically modified plant germplasms (Kim and Meagher, in preparation); 5) quantifying the abilities of transgenic cottonwood and rice to tolerate and process mercury in contaminated soil and water (Che *et al.*, 2003; Heaton *et al.*, 2003; Heaton *et al.*, 2005; Che *et al.*, 2006; Lyyra *et al.*, in prep.); and 6) initiating the first field testing of transgenic cottonwood on mercury contaminated sites (Ivry, 2004). We also continued to develop an important set of tools, biochemical assays and reagents essential to the phytoremedaition of mercury (Pawloski *et al.*, 2005).

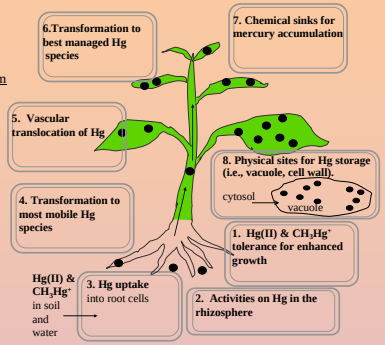
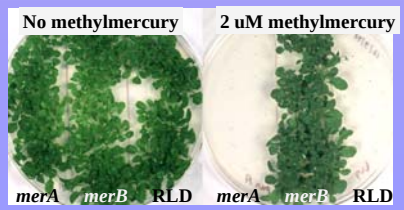


Figure 1. Molecular genetic strategies for mercury phytoremediation broken down into manageable foci for research. **1.** High-level tolerance to the two major toxic forms of mercury is essential if plant roots are to penetrate and extract mercury from heterogeneous, contaminated sediments. Several resistance strategies was explored in our earliest DOE EMSP funded research (**Figure 2 & 3,** and **Bibliography**). **2.** Plants help create their own rhizosphere by secreting various enzymes and small molecules and by adjusting soil pH. For example, root acidification of the soil should increase mercury mobility in soil and enhance uptake. This strategy has not yet been tested. **3.** The uptake of nutrients into plant roots and root hairs and movement through the apoplast requires the expression of membrane transporters. We predict that zinc, copper, and phosphate transporters mediate mercury uptake, movement, or exclusion (See **Figure 4**). **4.** Transformation of some toxic elements to different electrochemical states or chemical species is necessary to increase their rate of transport. For example, mercury can be transported as elemental mercury Hg(0) or ionic mercury Hg(II) (see **Bibliography**). **5.** Vascular movement of mercury as for exmple a zinc analog up from the roots via the zylem and distribution via the phloem is an important part of nutrient management, but xylem transport is poorly defined at the genetic level. We hope to enhance mercury xylem loading in roots and unloading in leaves. **6.** Many elements could be stored aboveground in a different electrochemical state or as a different chemical species than that which is ideally transported. For example, we are proposing to convert all mercury to Hg(II) in order that it can be stored in complexes with thiol-containing peptides and proteins. **7.** Chemical sinks, such as acidic chelators, amino acids, and thiol-reactive peptides, can bind Hg(II) **(Figure 5)**. **8.** Physical organellar sinks are needed as storage areas for mercury and these might include vacuoles, trichomes, and dead vascular elements. We have targeted the transport of thiolpeptide – mercury complexes into the vacuole for storage **(Figure 6, Bibliography)**.

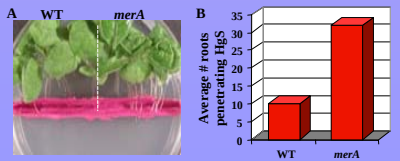
Strategy #1. Methyl-Hg resistance for maximum plant growth.

Figure 2. Constitutive expression of the methylmercury lyase gene (*merB*) in *Arabidopsis* confers strong resistance to methylmercury (Bizily et al., 1999, 2000, 2003). Similar results are obtained in tobacco and cottonwood (Unpublished and Che et al., 2006).



Strategy #1 (cont.). Hg(II) resistance for maximum root penetration into contaminated soils and through hotspots.

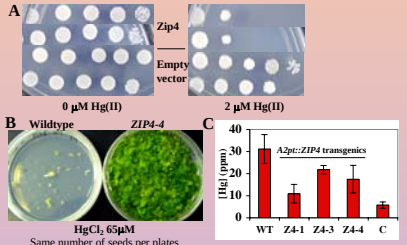
Figure 3. *MerA* tobacco roots penetrate a HgS barrier more efficiently than wild-type roots. *MerA* encodes the mercuric ion reductase. The average number of roots growing through a barrier of HgS is scored as a function of the *merA* or WT genotype. **A.** Wildtype (WT) and *merA* tobacco seeds were germinated and grown on a vertical plate toward a barrier of HgS. Roots observed from the bottom of the plate were traced back to WT or *merA* plants at the top. **B.** The *merA* roots are 3 to 4 times more likely to penetrate the toxic HgS barrier than WT. The same number of WT and merA seedings were germinated on each half of the plate.



Strategy #3. Increasing mercury uptake as a zinc nutrient analog.

Mercury is a chemical analog of zinc. Zinc is taken up into roots and moved throughout the apoplast via a family of high affinity transporters (ZIPs). ZIPs are eight membrane pass integral membrane proteins. Arabidopsis has a family of 11 ZIPs. We tested the ability of each ZIP (supplied from Mary Lou Guerinot, Dartmouth) to uptake mercury screening for a mercury sensitive phenotype first in yeast. Those ZIPs producing a yeast mercury sensitive phenotype (ZIP4 and ZIP7) were retested in transgenic *Arabidopsis*.

Figure 4. ZIP overexpression in yeast and plants. ZIP4 overexpression conferred strong mercury sensitivity to yeast (**Figure 4A**), but surprisingly conferred mercury resistance to Arabidopsis (**Figure 4B**). It appears ZIP4 pumps mercury out of plants (**Figure 4C**). ZIP7 expression makes both yeast and plants mercury sensitive (not shown) and we will go on to examine mercury accumulation in ZIP7 overexpressing plants.



Strategy #7. Increasing mercury sinks.

Phytochelatins (PCs) are synthesized in three steps from common amino acids via the tripeptide glutathione (GSH) as shown in **Figure 5A**. GSH and PCs can sequester Hg(II) (**Figure 5B**) and may enhance Hg transport into the vacuole. We tested the ability of of all three enzymes to enhance mercury resistance and accumulation. Expression of ECS (ECS11, **Figure 5D**) and PCS (**Figure 5C**) each enhanced resistance. Only the expression of ECS and GS together produced both resistance and accumulation (ECSxGS, **Figure 5D & E**).

Figure 5. Overexpression of ECS and GS together enhance mercury resistance and aboveground accumulation.

A

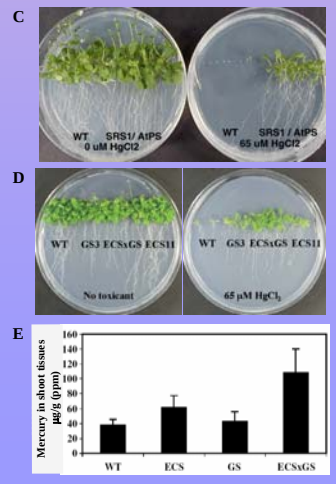
γ-EC glutathione (GSH) PCs

Glu + Cys → γ-GluCys → Gly g-GluCys → Gly(g-GluCys)$_n$
ECS GS PCS

ECS = g-glutamylcysteine synthetase
GS = glutathione synthetase
PCS = phytochelatin synthase, n = 2-11

B

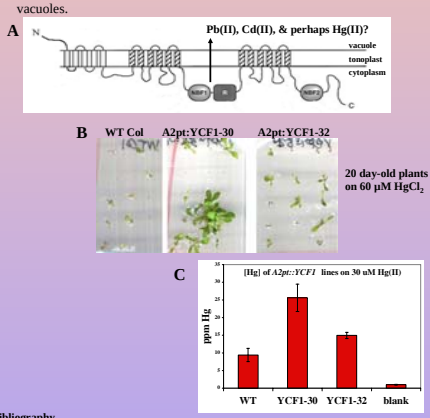
$GSH + Hg(II) \rightarrow Hg\text{-}(GS)_2 + 2H^+$



Strategy #8. Increase Vacuolar Sequestration

Our strategy is to enhance the vacuolar storage of Hg(II) to help construct a mercury hyperaccumulator. There is a solid base of literature confirming the vacuolar storage of glutathione conjugates of toxic chemicals in yeast and animals and plants. The subclass of ABC transporters that function as vacuolar glutathione conjugate pumps (GCP) was named MRPs for multi-drug resistance pumps after their first discovery in multi-drug resistant human cancer cell lines that resisted therapeutic drug treatment. Both human MRPs and plant MRPs are closely related in domain structure to the well-characterized yeast YCF1 MRP (YCF1) (**Figure 6A**). This domain structure sets them apart from other classes of ABC transporters. Among the family of approximately 130 ABC transporters in *Arabidopsis*, there is a subclass of 15 genes encoding predicted vacuolar GCPs. We are examining the utility of the 15 *Arabidopsis* MRPs and yeast YCF1 in mercury transport into the vacoule. Yeast YCF1 appears to significantly enhance mercury resistance and likely transport into plant vacuoles for storage (**Figure 6**).

Figure 6. Glutathione (GSH) conjugate pumps like yeast YCF1, pump GS-cadmium complexes across into vacoule and appear to act on mercury-complexes. A. Model of YCF1 in the membrane. **B.** Mercury resistance of transgenic *Arabidopsis* lines segregating a yeast YCF1 expression construct. A construct *A2pt::YCF1* expressing *YCF1* from the strong vegetative actin *ACT2* promoter was transformed into plants. Among 50 independently transformed plants lines we screened out 2 lines (-30 and -32) that were most resistant to mercury. **C.** These two *A2pt::YCF1* lines accumulated more mercury than WT, presumably because they sequester more Hg(II)-GS conjugates in their vacuoles.



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