Strategies for the engineered phytoremediation of mercury and arsenic pollution

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Introduction

Mercury and arsenic are extremely toxic heavy metal and metalloid pollutants that adversely affect the health of millions of people worldwide [1]. Mercury and arsenic have reached unacceptably high levels in the environment due to industrial, defense, agricultural, and municipal properties. Hundreds of Superfund sites in the United States are listed on the National Priority List (NPL) (www.epa.gov/superfund/sites/nl/info.html) as having unacceptably high levels of mercury and/or arsenic and are recommended for cleanup. In the majority of cases, these sites are not remediated because of the high costs associated with physical methods of cleanup. Physical remediation methods involving soil removal and reburial are expensive, impractical on the scale that is needed, and environmentally destructive. Higher plants can extract pollutants from soil or water through their normal root uptake of nutrients. They can store and concentrate pollutants in cells and/or convert toxic pollutants to less toxic forms [2]. A plant-based phytoremediation approach to heavy metals uses plant roots to extract, the vascular system to transport, and the leaves as a sink to concentrate the elements above-ground for harvest and processing.

Our working hypothesis is that controlling the electrochemical state of mercury and arsenic in aboveground tissues and/or increasing thiol sinks throughout the plant will result in both resistance to and hyperaccumulation of mercury and arsenic. Protonolysis of methylmercury to ionic mercury (Hg(II)) which is highly thiol reactive, favors the trapping of mercury as Hg(II)-thiol complexes in the aboveground tissues. Electrochemical reduction of Hg(II) to metallic mercury (Hg(0)) results in volatilization of the metal from the plants. Most arsenic in surface soil and water exists primarily in its oxidized form, the oxyanion arsenate (AsO_4^{-3}). Arsenate, which is an analogue of phosphate, can potentially be taken up from soil and translocated up the plant vascular system along with phosphate [3]. In contrast, the reduced form of arsenic, arsenite (AsO_3^{-3}), has a strong affinity toward thiol groups and once formed aboveground in leaf and stem tissues should be trapped as peptide-thiol complexes [4].

Several strategies for the phytoremediation of arsenic and mercury pollution, using bacterial, animal, and plant genes, have been tested in model plants including *Arabidopsis* and tobacco. We have demonstrated that plants can be engineered to take up and tolerate several times the levels of mercury and arsenic that would kill most plant species. We have engineered plants that detoxify, volatilize or sequester these elements [5-7]. Using these modified plants, methylmercury and/or ionic mercury is detoxified, stored below or above ground, and even volatilized as part of the transpiration process, and thus keeping it out of the food chain. Initial efforts with arsenate demonstrate that it can be taken up, transported above-ground, electrochemically reduced to arsenite in leaves, and sequestered in thiol-rich peptide complexes

[7]. Transgenic mercury remediation strategies also worked efficiently in cultivated and wild plant species like canola, rice, yellow poplar and cottonwood. Genomic surveys suggest that thousands of other bacterial, animal, and plant genes are available to help us enhance these strategies in the near future.

Results and Discussions

Mercury detoxification and remediation

Our laboratory made use of two genes from the well-characterized bacterial *mer* operon, *merA* and *merB*, in order to engineer a mercury transformation and remediation system in plants [5, 6]. The bacterial *merB* gene encodes an organomercury lyase that is responsible for the protonolysis of compounds like MeHg into reduced organic molecules (in this case methane) and ionic mercury Hg(II) as shown in Reaction #1. The bacterial *merA* gene encodes an NADPH-dependent mercuric ion reductase that converts ionic mercury (Hg(II)) to elemental, metallic mercury (Hg(0) (Reaction #2). Hg(0) is nearly two orders of magnitude less toxic than ionic mercury and is readily eliminated by plants due to its volatility.

MerB catalyzed	$CH_3Hg^+ + H+ \rightarrow CH_4 + Hg(II)$	**Reaction #1**
MerA catalyzed	$Hg(II) + NADPH + OH^- \rightarrow Hg(0)a + NADP^+ + H_20$	**Reaction #2**

MerA plants confer strong resistance to ionic mercury

We successfully engineered model *Arabidopsis thaliana* and tobacco plants to use a highly modified bacterial mercuric ion reductase gene, *merA*, to further detoxify ionic mercury (Hg(II)), electrochemically reducing it to Hg(0). These plants germinate, grow, and set seed at normal growth rates on levels of Hg(II) that are lethal to normal seeds and plants [5]. These Hg(II) levels are in excess of what is present at 90% of most contaminated sites (1-500 ppm). For example, figure 1 shows the result of transplanting wild-type (left of each pair of pots) and *merA* (right of each pair) expressing tobacco plantlets into heavily mercury-contaminated soil. The *merA* plants perform exceedingly well. In five replications of this experiment, 100% of the *merA* plants achieve full size and seed set, while the controls all die shortly after planting. Our MerA expressing *Arabidopsis* and tobacco plants thrive on heavily mercury contaminated medium that kills wild-type plants.

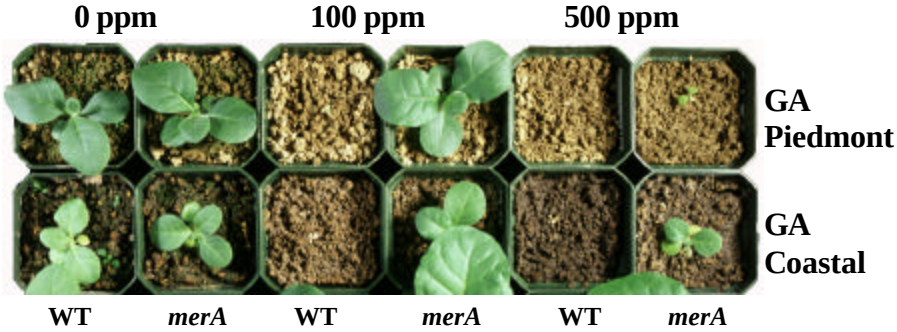

Figure 1. MerA confers strong resistance to ionic mercury in soil. Three-week-old wild-type (WT) tobacco plantlets and transgenic plantlets expressing *merA* were transplanted into two different native Georgia soils (GA) without (0 ppm) or with mercury at the concentrations indicated. This photograph was taken three weeks later. The WT controls died on mercury and are indicated by arrows.

MerB plants confer strong resistance to ionic mercury

Next, we engineered *Arabidopsis* to use a slightly modified bacterial *merB* gene, methylmercury lyase, to convert methylmercury to much less toxic ionic mercury. These plants germinate, grow, and set seed at normal growth rates on levels of methylmercury that are lethal to normal plants, as shown in Figure 2 [6]. This 100-fold range in concentrations (0.01-2 ppm) far exceeds concentrations commonly found in the environment (0.01-0.2 ppm). Plants expressing both MerA and MerB proteins detoxify methylmercury in two steps to the least toxic metallic form Hg(0) [8]. These plants are even more resistant to methylmercury (>10 ppm) than plants expressing MerB alone.

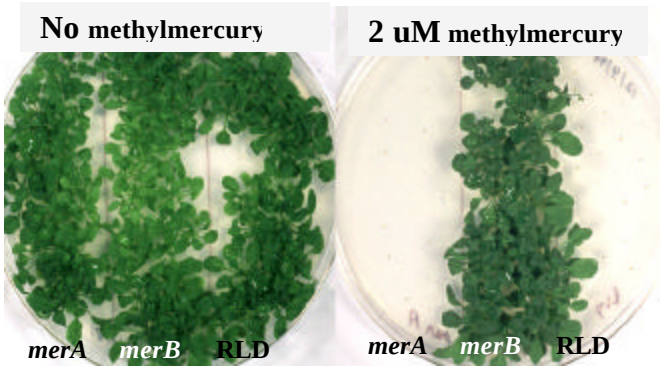

Figure 2. MerB expressing *Arabidopsis* are resistant to methylmercury. Seeds from wild-type control (RLD), *merA*, and *merB* expressing lines were germinated on 0.5x MS medium without (left) and with 2 µM methylmercury and grown for three weeks. Only *merB* expression allows normal growth on methylmercury.

Arsenic detoxification and hyperaccumulation

The most efficacious remediation of arsenic requires that plants extract arsenic from soil and hyperaccumulate it aboveground in leaves and stems. To accomplish this we must change the electrochemical species of arsenic in different parts of the plant. The bacterial *ArsC* gene encodes a 141 amino acid arsenate reductase, ArsC (Accession #J02591) and uses glutathione (GSH) as hydrogen/electron donor in the electrochemical reduction of the oxyanion arsenate (AsO_4^{-3}), to the oxyanion, arsenite (AsO_3^{-3}) [9]. While arsenate is a phosphate analogue, arsenite is chemically very different, being a highly reactive species with strong affinity toward thiol-groups such as those in *g*-EC, GSH, and PCs (Figure 3).

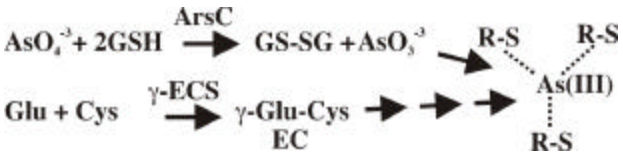

Figure 3. ArsC and g-ECS catalyzed reactions. The bacterial arsenate reductase (ArsC) catalyzes the electrochemical reduction of arsenate to arsenite. The bacterial γ-glutamylcysteine synthetase (γ-ECS) catalyzes the formation of γ-glutamylcysteine (γ-EC) from the amino acids glutamate and cysteine and is the committed step in the synthesis of glutathione (GSH) and phytochelatins, PCs (indicated by three arrows). Reduced arsenite can bind organic thiols (RS) such as those in γ-EC, GSH, and PCs through the replacement of oxygen by organic sulfur species.

We co-expressed two bacterial genes, *arsC* and *g-ECS*, under control of a light-regulated, leaf-specific rubisco small subunit promoter (*SRS1p*), and a constitutively expressed actin promoter/terminator cassette (*ACT2pt*) [7]. Transgenic plants expressing bacterial ArsC were hypersensitive to arsenate (Figure 4 right lane). This is undoubtedly due to the reduction of arsenate to more toxic arsenite in leaves. Plants expressing *g*-ECS were moderately resistant to arsenate (Figure 4 left lane) whereas, doubly transformed plants expressing ArsC and *g-ECS* together were significantly resistant to arsenate (Figure 4 middle lane). After three weeks of growth on 200 µM arsenate, the hybrid plants (ArsC9 +*g*-ECS) attained three-fold more biomass than plants with *g*-ECS alone, six-fold more than wild-type, and ten-fold more than ArsC alone. Additionally, when the plants were allowed to grow for up to four weeks, the hybrid plants accumulated four-fold more biomass than γ-ECS alone and approximately 17-fold more than ArsC alone [7]. For determining the total arsenic concentrations in shoots, two hybrid lines expressing ArsC9 and γ-ECS together and lines expressing SRS1p/ArsC9 or ACT2p/ECS1 alone, and wild-type controls were grown on 125 µM arsenate for three weeks. Both hybrid lines expressing ArsC9 + γ-ECS showed three-fold higher concentrations of arsenic in their shoots than wild type, ACT2p/ECS1, and SRS1p/ArsC9 plants alone.

200 mM Arsenate

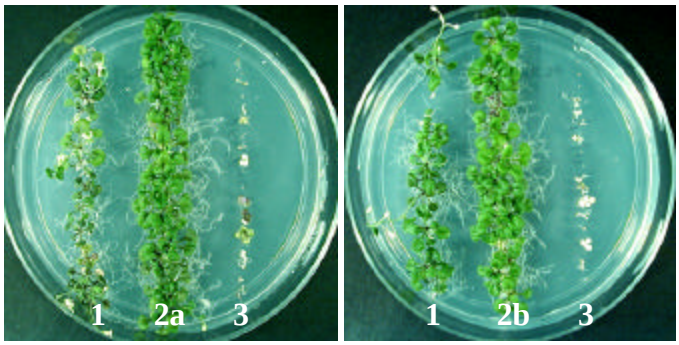

Figure 4: Arsenic resistance of plants expressing ArsC9 and g-ECS. Each arsenate containing plate has a column of seeds from plants that constitutively express (1) ACT2p/γECS/ACT2t (left); (2a, 2b) doubly transformed lines expressing both transgenes, γECS and ArsC (center); and (3) light regulated SRS1p/ArsC/Nost (right) grown on 200 µM sodium arsenate for four weeks. Wild type (not shown), showed intermediate resistance between the two lines with single transgenes. Seeds were germinated and grown for four weeks on 200 µM arsenate containing 1/2 strength MS media.

These results can be explained as follows. The coexpressed *SRS1p/ArsC* and *ACT2p/ECS* transgenes complemented each other's activities resulting in increased arsenic resistance. The *g-ECS* gene product directs the synthesis of the dipeptide *g* -EC, the first and proposed limiting enzyme in the phytochelatin (PC) synthetic pathway [10]. All downstream thiol-peptide compounds in the phytochelatin pathway (e.g., *g*-EC, GSH, and PCs) can bind arsenite and potentially contribute to arsenic tolerance and accumulation. *ACT2pt* driven constitutive expression of γ-ECS enzyme should have resulted in increased thiol-sink peptides in all major vegetative organs (e.g., roots, stem, leaves, petals, sepals) [11] and result in plants that can grow on arsenic contaminated media. In addition, the overexpression of both ArsC and *g*-ECS proteins together in hybrid plants further enhanced arsenic tolerance and hyperaccumulation of arsenic in the aboveground parts, far beyond what *g*-ECS expression could achieve alone. We suggest this is due to more extensive electrochemical reduction of arsenate to arsenite by the activity of ArsC

and binding of arsenite to the immediate or downstream products of *g*-ECS (e.g. *g*-EC, GSH, PCs). A similar combined strategy is being used to enhance mercury hyperaccumulation. Several other strategies are also being examined to enhance the extraction, transport, and storage of mercury and arsenic. Initial fields testing of existing mercury strategies gave promising results and are being expanded.

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