Genetic engineering to enhance mercury phytoremediation
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Most phytoremediation studies utilize merA or merB genes to modify plants via the nuclear or chloroplast genome, expressing organomercurial lyase and/or mercuric ion reductase in the cytoplasm, endoplasmic reticulum or within plastids. Several plant species including Arabidopsis, tobacco, poplar, rice, Eastern cottonwood, peanut, salt marsh grass and Chlorella have been transformed with these genes. Transgenic plants grew exceedingly well in soil contaminated with organic (∼400 µM PMA) or inorganic mercury (∼500 µM HgCl₂), accumulating Hg in roots surpassing the concentration in soil (∼2000 µg/g). However, none of these plants were tested in the field to demonstrate real potential of this approach. Availability of metal transporters, translocators, chelators and the ability to express membrane proteins could further enhance mercury phytoremediation capabilities.

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Phytoremediation is the use of plants to clean up contaminated environments. It is a cost-effective, environmentally friendly approach with great advantages for large scale clean-up of contaminated sites [1–3]. Current remediation methods for heavy metal contamination are environmentally invasive, expensive and inefficient, especially for large scale clean-up [4]. However, plants have limited capabilities for treating high levels of heavy metal contamination.

Mercury (Hg), one of the most toxic pollutants threatening our health and ecosystems, has been introduced from natural and anthropogenic sources. Recent estimates calculate the annual global emissions between 4800–8300 tons per year [5]. In the United States alone coal burning power plants emit about 48 tons of mercury annually [6]; the combined estimates for Asia and Africa surpass 1500 tons [7]. The cost of remediation of each pound of mercury from the environment using current technologies is in the range of tens of thousands of dollars. Therefore, finding alternate remediation approaches is an urgent need.

Mercury is usually released in the metal or ionic form, accumulating in sediments where it becomes methylated by anaerobic sulfate-reducing bacteria to produce methyl mercury, a highly toxic organomercurial compound. The toxicity of mercury greatly depends on the form present in the environment. Inorganic mercury forms are usually less harmful than organic forms, partly because they bind strongly to soil components that reduce their availability and absorption. On the contrary, organomercurials are highly toxic because of their hydrophobicity, which facilitates their movement across cell membranes and accumulation in membrane bound organelles, inhibiting essential oxidative and photosynthetic pathways. The toxic effects of methyl mercury are further magnified at higher trophic levels owing to increased accumulation in tissues [8,9]. Organomercurials are potent neurotoxins with over 90% absorption into the blood stream from the intestinal tract, while mercury salts and elemental mercury show reduced absorptions of less than 10% and 0.1%, respectively [10]. In plants, ionic mercury tends to affect the plasma membrane where it damages membrane transporters such as aquaporins, leading to nutrient and water disruption [11]. Organomercurials rapidly localize to plastids where they accumulate and disrupt important metabolic functions including electron transport, oxygen evolution [12], photophosphorylation, Hill reaction, chlorophyll content and chlorophyll fluorescence [13,14].

Plants cannot successfully detoxify or interconvert mercury to more benign forms. Genetic engineering can integrate genes from other organisms to enhance phytoremediation capabilities in plants. This article reviews our current knowledge of transgenic plant systems for Hg phytoremediation, provides insight on mechanistic aspects and directions for further studies.

MerAB engineered via the nuclear genome
A well characterized phytoremediation system is the use of the bacterial merA (mercuric ion reductase) and merB (organomercurial lyase) genes to genetically engineer plants for the remediation of Hg [15–26]. Organomercurial lyase facilitates protonolysis of organic-Hg to Hg²⁺ while mercuric ion reductase reduces Hg²⁺ to Hg⁰, which is volatilized from plants. Initial attempts to use the
native bacterial merA gene in nuclear transgenic plants failed to express the mercuric ion reductase [24, 25]. When the coding sequence was modified to plant preferred codons, transgenic Arabidopsis thaliana, yellow poplar, peanut and Eastern cottonwood plants were resistant to levels up to 25–100 μM HgCl₂ [24, 25, 26]. Kim et al. [22] demonstrated that root-specific expression of the merA gene in Arabidopsis thaliana provided resistance up to 80 μM HgCl₂ (Table 1). Heaton et al. [27] showed that the root of merA transgenic plants grew directly into Hg ‘hotspots’ while the wild type grew away from the source. These results indicated that root protection was important for phytoremediation and that root is the main organ affected by Hg. One drawback of this finding was the implication that Hg was being detoxified to Hg⁰ in the root where it may become volatilized, though Hg⁰ could also be transported through the xylem to the leaves where it is transpired.

Heaton et al. [20] developed merA transgenic rice (Oryza sativa) resistant to 250 μM HgCl₂; the wild type was resistant up to 150 μM HgCl₂. This was the first report of a transgenic monocot for phytoremediation of Hg. Also, salt marsh cordgrass (Spartina alterniflora) modified for Hg phytoremediation was resistant up to 500 μM HgCl₂ [19]. It should be pointed out that both these monocots showed natural tolerance to Hg and further studies are needed to understand the mechanism of such resistance. Recently, Huang et al. [7] demonstrated that transgenic Chlorella expressing the merA gene withstood up to 40 μM HgCl₂. Microalgae have certain advantages over land plants for phytoremediation, including larger surface area in contact with the contaminant in water, and a natural capacity to bind Hg [28].

Unfortunately, mercuric ion reductase did not protect against the more toxic and environmentally relevant organic-Hg. Both the merA and the merB genes are needed to protect cells from organic-Hg. When a codon optimized merB was used to transform the Arabidopsis thaliana nuclear genome, it provided resistance up to 1 μM phenylmercuric acetate (PMA) [17]; although this is a very low level of resistance, this concentration was lethal to untransformed plants. The organic-Hg concentration needed to inhibit growth in untransformed plants was 100-fold less than the level required for HgCl₂, confirming higher toxicity. Transgenic Eastern cottonwood trees expressing merB failed to confer resistance to PMA [18].

In 2000, Bizily et al. [16] reported the first transgenic plant expressing both the merA and merB genes; Arabidopsis thaliana transgenic lines showed resistance up to 5 μM PMA and 10 μM CH₃Hg, a fivefold increase in resistance when compared with transgenic plants expressing only the merB genes. Transgenic Eastern cottonwood trees expressing both genes were tolerant up to 10 μM of PMA [23]. Bizily et al. [15] showed that transgenic plants in which organomercurial lyase was localized to the endoplasmic reticulum (ER) and to the cell wall (CW) conferred as much resistance (up to 5 μM PMA) as transgenic plants expressing both genes in the cytoplasm. Organomercurial lyase localized to the ER showed 41 times as much activity as organomercurial lyase in the cytoplasm, suggesting that organic-Hg may be localized to membrane bound organelles (Table 1).

**MerAB engineered via the chloroplast genome**

In order to provide higher levels of resistance (>5–10 μM PMA), the plastids may need protection from toxic mercury. There are different types of plastids present in plant organs and tissues. Chloroplasts present in green tissues are metabolically very active and are involved in photosynthesis, sulfur/nitrogen metabolism and synthesis of starch, amino acids, fatty acids, pigments, vitamins and so on. Chloroplasts are the primary source of world’s food and oxygen, sustaining life on this planet. Other types of plastids include colourful chromoplasts present in flowers, fruits and vegetables. Amyloplasts are present in tubers like potato or in endosperm tissues in seeds, primarily performing the function of storage. Non-green proplastids are present in roots and developing tissues. In spite of variation in their appearance and functions, all plastids contain identical genome structure and sequence.

The idea of protecting essential metabolic reactions occurring within plastids, has been our working hypothesis for expressing merA and merB genes within plant chloroplasts [29]. Chloroplast has been shown to be the main target for Hg poisoning [12, 30, 31]. Transgene containment by the maternal inheritance of the plastid genome [32] or cytoplasmic male sterility [33] is another major advantage of the chloroplast transformation system. Chloroplast genetic engineering is especially advantageous for the development of transgenic plants when multiple genes are required for effective phytoremediation [34]. By using chloroplast transformation, multigenetic pathways can be developed in a single transformation event without the need for backcrosses of independent lines or re-transformation. Because plastids have retained the genetic machinery from bacterial ancestor, their genomes can transcribe and translate operons [34]. Chloroplast genetic engineering also circumvents common drawbacks of nuclear transformation, including gene silencing and position effect. Site-specific transgene integration by homologous recombination [35] eliminates the position effect. High expression levels, up to 47% of total leaf protein in healthy plants [36] or accumulation of transcripts 150-fold higher than nuclear transgenic plants [37] did not result in post-transcriptional or post-translational gene silencing. Such high expression levels are achieved because there are up to 10 000 copies of transgenes in each transformed plant cell. Also, the addition of transcriptional and translational enhancer elements is needed to optimize expression of transgenes.
Table 1

Advances in transgenic systems for phytoremediation of mercury.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Expression compartment</th>
<th>Level of resistance</th>
<th>Mode of resistance</th>
<th>Special feature</th>
<th>Year</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. thaliana</td>
<td>merA</td>
<td>Cytoplasm</td>
<td>100 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>First report of merA in plants</td>
<td>1996</td>
<td>[25]</td>
</tr>
<tr>
<td>Yellow Poplar (L. tulipifera)</td>
<td>merA</td>
<td>Cytoplasm</td>
<td>50 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>merA transformed into a tree species</td>
<td>1998</td>
<td>[24]*</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>merB</td>
<td>Cytoplasm</td>
<td>2 μM PMA</td>
<td>PMA to Hg(II)</td>
<td>First report of merB in plants</td>
<td>1999</td>
<td>[17]</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>merA/B</td>
<td>Cytoplasm</td>
<td>5-10 μM organic-Hg</td>
<td>PMA to Hg(II) to Hg(0)</td>
<td>First report of merA and merB in plants</td>
<td>2000</td>
<td>[16]**</td>
</tr>
<tr>
<td>Tobacco (N. tabacum)</td>
<td>merA</td>
<td>Cytoplasm</td>
<td>50 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>merA transformed into tobacco</td>
<td>2001</td>
<td>[21]</td>
</tr>
<tr>
<td>Tobacco</td>
<td>merA/B</td>
<td>Plastid</td>
<td>400 μM PMA</td>
<td>PMA to Hg(II) to Hg(0)</td>
<td>First chloroplast phytoremediation system</td>
<td>2003</td>
<td>[29]**</td>
</tr>
<tr>
<td>Tobacco</td>
<td>merB</td>
<td>Endoplasmic reticulum (ER)</td>
<td>5 μM PMA</td>
<td>PMA to Hg(II)</td>
<td>MerB targeted to the ER</td>
<td>2003</td>
<td>[15]</td>
</tr>
<tr>
<td>Rice (O. sativa)</td>
<td>merA</td>
<td>Cytoplasm</td>
<td>250 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>First monocot for Hg phytoremediation</td>
<td>2003</td>
<td>[20]</td>
</tr>
<tr>
<td>Eastern cottonwood</td>
<td>merA</td>
<td>Cytoplasm</td>
<td>25 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>merA transformed into a forest tree</td>
<td>2003</td>
<td>[18]</td>
</tr>
<tr>
<td>(P. deltoides)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut (A. hypogaea)</td>
<td>merA</td>
<td>Cytoplasm</td>
<td>100 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>merA transformed into peanut</td>
<td>2003</td>
<td>[26]</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>merA</td>
<td>Cytoplasm of root cells</td>
<td>80 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>Root-specific expression of merA</td>
<td>2005</td>
<td>[22]**</td>
</tr>
<tr>
<td>Salt marsh cordgrass (S. alterniflora)</td>
<td>merA</td>
<td>Cytoplasm</td>
<td>500 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>First wetland grass for Hg phytoremediation</td>
<td>2006</td>
<td>[19]</td>
</tr>
<tr>
<td>Chlorella</td>
<td>merA</td>
<td>Cytoplasm</td>
<td>40 μM HgCl₂</td>
<td>Hg(II) to Hg(0)</td>
<td>First transgenic alga for Hg bioremediation</td>
<td>2006</td>
<td>[7]</td>
</tr>
<tr>
<td>Tobacco</td>
<td>ppk</td>
<td>Cytoplasm</td>
<td>10 μM HgCl₂</td>
<td>Hg chelation</td>
<td>First use of a Hg-scavenging agent</td>
<td>2006</td>
<td>[48]**</td>
</tr>
<tr>
<td>Eastern cottonwood</td>
<td>merA/B</td>
<td>Cytoplasm</td>
<td>10 μM PMA</td>
<td>PMA to Hg(II) to Hg(0)</td>
<td>merA and merB transformed into a forest tree</td>
<td>2007</td>
<td>[23]</td>
</tr>
<tr>
<td>Tobacco</td>
<td>merA/B</td>
<td>Plastid</td>
<td>300 μM PMA/HgCl₂</td>
<td>PMA to Hg(II) to Hg(0)</td>
<td>100-fold increase in Hg translocation and accumulation</td>
<td>2007</td>
<td>[44]**</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>merP</td>
<td>Cell membrane</td>
<td>10 μM HgCl₂</td>
<td>Possibly Hg(II) chelation</td>
<td>Localization to the cell membrane</td>
<td>2009</td>
<td>[49]**</td>
</tr>
</tbody>
</table>

PMA: phenylmercuric acetate; organic-Hg: PMA and methyl mercury; MerA/B: expression of merA and merB genes; references in bold: relevant.
However, there is no need for codon optimization of bacterial genes for their expression in plastids [29**].

In spite of these advantages, there are a few limitations of the chloroplast transformation technology. Unlike transformation of the nuclear genome of different crop species using the same transformation vectors, species-specific vectors are necessary for chloroplast transformation. Therefore, it is important to have the chloroplast genome sequence, especially the intergenic spacer regions and endogenous regulatory sequences (promoter, 5’UTR, 3’UTR) to create species-specific chloroplast vectors. Until 2004, complete genome sequences were available for only six crop species. However, plastid genome sequences are becoming available at a rapid pace, facilitating the application of chloroplast genetic engineering to more species than ever before [38–42]. The second challenge is achieving homoplasy (integration of transgenes into each chloroplast genome and elimination of untransformed chloroplast genome). This has been easily accomplished in plant species that are regenerated via organogenesis, after several rounds of selection. Chloroplast genomes of more than 10 crop species have been transformed via organogenesis, including cauliflower, lettuce, cabbage, petunia, poplar, tobacco, potato, tomato and so on [43]. However, achieving homoplasy in crops regenerated via somatic embryogenesis is challenging although carrot, cotton and soybean chloroplast genomes have been transformed already, with desired agronomic traits [43]. The versatility of the chloroplast system has led to its application in developing transgenic plants with enhanced agronomic traits [43]. Therefore, chloroplast genetic engineering is an ideal approach for mercury phytoremediation [29**].

In these studies, the native bacterial merA and merB genes were integrated into the tobacco chloroplast genome [29**,44**]. The transgenic plants were resistant to very high concentrations of PMA, up to 400 μM. This is the only report to date in which transgenic plants not only survived such high concentrations of PMA but also showed better growth than control untreated plants [29**]. Transgenic plants grew exceedingly well in soil contaminated with 300 μM PMA or HgCl₂, accumulating Hg in the root to levels surpassing the concentration in soil, up to 2000 μg/g [44**]. There are several reasons for successful phytoremediation via the chloroplast genome. Mercury, especially in the organic form, is targeted to the chloroplast. Mercuric ion reductase functions better in chloroplasts because of the abundance of NADPH. Protection of the root in the transgenic plant was possible because root proplastids are active and express the mer genes under the control of a constitutive promoter. Kumar et al. [45] showed that root proplastids expressed up to 70% of foreign protein as leaf chloroplasts. Interestingly, transgenic plants were able to accumulate 100-fold and 4-fold more Hg in the shoot in the presence of PMA or HgCl₂ than untransformed plants. This shows that organic-Hg is transported more efficiently than inorganic forms. Finally, the transgenic plants were shown to volatilize Hg⁰ efficiently, independent of the form of Hg in the soil, confirming that both mercuric ion reductase and organomercurial lyase were active in transgenic chloroplasts [44**].

Other mechanisms for phytoremediation of mercury

The mer operon has three known membrane transporter genes involved in the process of translocating Hg⁰ into the cell: merC, merP and merT [46,47]. Recently, Sasaki et al. [48**] showed that expression of the merC gene in Arabidopsis thaliana enhanced hypersensitivity to otherwise sublethal concentrations of HgCl₂. Therefore, plants can be genetically modified with bacterial metal transporters to enhance heavy metal uptake. In a subsequent report, Arabidopsis thaliana transformed with the merP gene showed resistance to 10 μM HgCl₂ instead of becoming hypersensitive [49**]. Because merP requires merT for Hg²⁺ translocation, it is possible that merP was trapping Hg at the cell membrane, thereby protecting the cytoplasm. To take advantage of these transport mechanisms, transgenic plants should be modified with the merT gene as well; this would provide resistance against higher levels of transported Hg. Alternatively, the merC can be coupled to a chelator gene like polyphosphate kinase (ppk) or metallothionein (mt), to develop transgenic plants that could accumulate Hg. Recently, it has been shown that by using chloroplast genetic engineering, membrane proteins can be targeted to the chloroplast inner envelope membrane and accumulated at very high levels [50**]. This opens the possibility of transforming the chloroplast genome with mer transporters to enhance Hg accumulation or phytoremediation.

One public concern regarding the use of the merAB system is the release of Hg⁰ into the atmosphere. Therefore, an alternative approach would be the sequestration of ionic mercury inside the cell by binding it to a chelating molecule. The bacterial ppk gene coding for polyphosphate kinase synthesized polyphosphates, which are negatively charged; the long phosphate polymers reduced cytotoxicity of heavy metals by chelation [51]. Evidence for this was first provided when nuclear transgenic tobacco plants expressing the ppk gene showed enhanced tolerance and accumulation of Hg²⁺ when grown in 10 μM HgCl₂ [52,53**]. Although the levels of resistance were low, the possibility of using metal-scavenging molecules for phytoremediation was demonstrated.

Future prospects for mercury phytoremediation

It is clear that plants can be genetically modified to enhance their tolerance, uptake, translocation, accumulation and volatilization capabilities for Hg phytoreme-
Several general conclusions can be drawn from the discussed reports. Transgenic plants grow exceedingly well in soil contaminated with PMA or HgCl₂, accumulating Hg in the root to levels surpassing the concentration in soil, up to 2000 μg/g or volatilizing mercury supplied in organic or inorganic form. However, none of these plants were tested in the field to demonstrate real potential of this approach. Availability of metal transporters, translocators and chelators could further enhance phytoremediation capabilities. Our recent ability to express membrane proteins opens the possibility of transforming the chloroplast genome with mer transporters to enhance Hg accumulation or phytoremediation. Alternatively, the merC can be coupled to a chelator gene like polyphosphate kinase (ppk) or metallothionein (mt), to develop transgenic plants that could accumulate Hg.

Plants tend to accumulate most mercury in roots, and translocation to shoot tends to be a major limitation, dependent upon concentration and cellular integrity [20,27,44**]. It seems that for translocation to occur, saturation of the roots to levels only withstood by transgenic plants is required. A recent report of a 100-fold increase in Hg translocation to the shoot [44**] is quite encouraging. However, in order to utilize the large biomass above ground, the translocation problem should be addressed further. Other interesting observations include promotion of growth in mer transgenic lines compared to untreated controls and increased chlorophyll content when grown in the presence of mercury [25,29**,54].

Conclusions
Toxicity of methyl mercury is magnified owing to its high accumulation in tissues; over 90% of methyl mercury is absorbed into the blood stream from the gastrointestinal tract when compared with 0.1% of elemental mercury [10]. Methyl mercury accumulated in fish is, therefore, 990-fold more neurotoxic than elemental mercury in thermometers. US EPA cautions pregnant women against frequent consumption of fish. Therefore, it is very important to develop technologies to clean up our environment. The potential of transgenic plants for Hg phytoremediation has been demonstrated, but important questions need to be answered to explore the full potential of this technology. A better understanding of Hg-forms transported and accumulated, and their saturation and steady-state levels in...
different tissues would help develop phytoremediation systems that are cost-effective. New mercury phytoremediation technologies would rely on different gene combinations to enhance uptake, translocation, chelation or detoxification and release of Hg⁰ into the atmosphere (Figure 1). Ultimately, the scientific community should make an effort to address the most important questions that deter the application of phytoremediation and produce plants with the best capabilities, including plants with large biomass, rapid growth rate, adaptation to wider climatic conditions and expression of multiple genes in different cellular compartments. The use of edible plant species should be avoided in phytoremediation applications, while plant species that outcross with wild relatives or cultivated plants may be used only when containment measures are applied including harvesting transgenic plants before the plant reproductive age, using a male sterility or maternal applied including harvesting transgenic plants before the plants may be used only when containment measures are

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

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** of outstanding interest


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Genetic engineering to enhance mercury phytoremediation


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32. Satab SC: Copper ion inhibition of electron transport activity in 


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44. Hussein H, Ruiz ON, Terry N, Daniell H: Phytoremediation of mercury and organomercurials in chloroplast transgenic plants: enhanced root uptake, translocation to shoots and volatilization. Environ Sci Technol 2007, 41:8439-8446. This report demonstrated that chloroplast transgenic plants expressing merA and merB can accumulate Hg in roots to levels surpassing the concentration in soil, up to 2000 μg/g without any detrimental effect and could accumulate 100-fold more Hg in leaves than untransformed plants.


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