ENHANCED MERCURY PROCESSING BY PLANTS GENETICALLY ENGINEERED FOR MERCURY PHYTOREMEDIATION

by

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(under the direction of Bruce Lee Haines)

ABSTRACT

Industrial practices have released mercury into soils and sediments that now require remediation. Current remediation strategies are costly, site-destructive and may cause a temporary increase in mobilization of mercury from contaminated sites. We are developing an alternative mercury remediation strategy, phytoremediation. Phytoremediation is the use of plants to extract, then sequester or detoxify contaminants from polluted soil or water. We have engineered several plant species with modified versions of the bacterial mercuric ion reductase gene, *merA*. The MerA enzyme electrochemically reduces Hg(II) to the less toxic and volatile Hg(0). Tobacco (*Tabacum nicotiana)*, rice (*Oryza sativa*) and cottonwood (*Populus deltoides*) plants engineered with the *merA* gene have a strong survival advantage over wildtype counterparts when grown on highly Hg(II)-contaminated liquid or soil media. Direct measurement of Hg(0) volatilization from *merA* rice plants confirms that resistance is a result of MerA-mediated electrochemical reduction of Hg(II) to Hg(0). A lower tissue retention of root-absorbed

Hg(II) in *merA* tobacco, rice and cottonwood than in wild-type counterparts provides further evidence of efficient Hg(II) reduction followed by Hg(0) volatilization. Tobacco plants were used as a model to study changes in mercury/plant interactions caused by the introduction of the *merA* gene. *merA* tobacco roots growing on semisolid growth medium penetrated an insoluble HgS barrier more effectively than wild-type roots. MerA activity provided partial protection from Hg(II)-induced transpiration stress in transgenic tobacco on $HgCl_2$-spiked hydroponics medium. Much lower leaf retention of atmospherically absorbed Hg(0) in *merA* than wild-type plants incubated in a closed chamber confirmed endogenous plant Hg(0) oxidation was efficiently countered by MerA Hg(II) reduction. Wild-type tobacco shoots grafted to *merA* tobacco roots accumulated and/or retained more root-absorbed mercury in aboveground tissues than intact *merA* or wild-type plants. This introduces the feasibility of engineering a mercury phytosequestering plant with root-specific *merA* activity. Results from experiments with *merA* tobacco, rice and cottonwood suggest that *merA* effectively mobilizes mercury within plants, allowing vertical mercury transport and efficient Hg(0) volatilization. Although the *merA* gene is likely to significantly enhance the ability of plant roots to encounter and absorb soil-bound Hg(II), additional engineering may be required for efficient soil Hg(II) extraction.

INDEX WORDS: *Nicotiana tabacum*, *Oryza sativa*, *Populus deltoides*, *merA*, MerA, Elemental mercury, Ionic mercury, Methylmercury, Hg(II), Hg(0), MeHg, Genetic engineering, Transgenic plants, Phytoremediation, Phytovolatilization, Mercuric reductase gene, Mercury pollution

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Officially, I started working toward this degree in late March of 1997 when I met Bruce Haines, Rich Meagher and Clayton Rugh. The three made such an impression on me that I was almost immediately certain this was the place I wanted to work on my degree. I can't say what impression I may have made on them, though Clayton's very mistaken but persistent memory of my showing up in a corduroy suit is somewhat telling. With some administrative creativity and some help from Dave Coleman of Ecology, I found myself an Ecology doctoral student with Bruce Haines of Botany as major professor, working on Rich Meagher's mercury phytoremediation project in the Genetics department. I have never met two people with greater enthusiasm for science than Bruce or Rich. Everybody in the Meagher lab has been of an incredible support to me. Clayton kept me motivated. Scott Bizily tried to make me more efficient. Anne-Marie has been a great friend. My committee: Rich, Bruce, Bob Teskey, Malcolm Sumner and Eugene Odum were dedicated to making my research work. I entered and left every committee meeting feeling excitement instead of the dread some associate with committee meetings. Sadly, Eugene passed away this August. After the initial surprise, I think most of us were just grateful we had worked with such an extraordinary man who did, after all, have as full a life as any of us could hope to have. I'm grateful to Scott Merkle of Forest Resources for filling the vacancy on my committee at relatively short notice.

I would never have been prepared for my work here if it were not for my time at Virginia Tech with John Cairns and his last graduate student, John Heckman. The two taught me that it's not enough to work hard and it's not enough to be a great thinker. You have to work hard and work smart. In reality, there is no good way to determine the true beginning of my work toward this degree. At best I can suggest that it was some time during my youth when my parents struggled to get across the ideas to me that work yielded results and that results yielded reward. There are truly no words to describe the level of genuine support my parents have given me in virtually every endeavor I have attempted.

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CHAPTER 1

INTRODUCTION

INTRODUCTORY STATEMENT

Since the beginning of the industrial revolution, humankind has made enormous technological and social progress leading to greater human health and longer mean life expectancies. This progress has come at an enormous energetic cost and many of the most widely used sources of energy (e.g. fossil fuels, nuclear power), either do cause or may cause the release of toxins into the environment. Furthermore, the increasingly specialized tools that technological advance has first created and then used are often composed of or used in conjunction with materials that are toxic to organisms. Their widespread use has made the exposure of organisms (including humans) to these materials almost inevitable, though obviously undesirable.

Mercury is a toxic element. It has been released into greater contact with organisms as a direct result of human energy processing and as an accidentally-released component of numerous widespread technologies. In general, strategies are employed to remove mercury from contact with those organisms that can conduct the toxin to humans, or upon which our ecosystems depend. Unfortunately, these strategies typically employ the use of energy sources and technologies similar to those that were originally responsible for releasing mercury and other toxins.

The purpose of the work described herein is to explore the use of a new technology to remove mercury from those environments where it is concentrated, its toxicity enhanced and its mobility into sensitive organisms increased. This technology is centered around plants which have been genetically engineered with one or more bacterial genes. These genes encode enzyme(s) that catalyze the alteration of mercury electrochemical form. When developed fully, this new strategy is intended to allow the detoxification and/or controlled translocation of mercury from locations where it may threaten human health or the integrity of ecosystems we may depend on. The use of plants for mercury remediation avoids energy sources and tools that themselves are further sources of chemical contamination. The work presented here is part of the initial investigation into the ability of the first prototype plants engineered for mercury remediation to process and translocate mercury in a controllable and desirable way. I predict that as the field of genetic engineering advances, engineered organisms will replace mechanical tools for many applications, including in the remediation of environmental pollution. These "clean technologies" will result in reductions to the release of toxic substances so inexorably linked to industrial processes yet so toxic to organisms.

LITERATURE REVIEW

Mercury toxicity

Mercury is not known to be essential to any metabolic process (Sigel and Sigel, 1997), thus, any mercury in an organism can be considered a contaminant. Ionic

mercury, Hg(II), derives its biotoxic nature from an extremely high sulfur affinity, enabling strong reactions with sulfur-containing peptides (Carty and Malone, 1979), and causing the disruption of cellular processes. In humans, Hg(II) is primarily toxic to the kidneys (Chang, 1979). Metallic mercury, Hg(0), is toxic if inhaled in sufficient quantities (Stopford, 1979), leading to chronic shortness of breath and lung fibrosis. Toxicity is likely a result of the conversion of Hg(0) to Hg(II) after absorption (Ogata and Aikoh, 1984). Similarly, plants which absorb high levels of Hg(0) through stomata in leaves may suffer toxic symptoms (Siegel et al., 1984) presumably as a result of Hg(0) conversion to Hg(II) in tissues (Du and Feng, 1983; Heaton et al., in prep.). Inorganic mercury (metallic or ionic) can cause acute toxic symptoms of pulmonary irritation, nausea, diahrrehea, muscle aches, fever and an elevated white blood count. However, chronic exposure to these forms is more serious, leading to neurological problems, paranoia, vision or hearing loss, gum disease, and renal damage (Stopford, 1979). For those not occupationally exposed to mercury, the primary mode of entry of inorganic mercury is from dental amalgams (Summers et al., 1993).

Methylmercury (MeHg) is considered more toxic than inorganic mercury because of a high stability combined with both lipid solubility and ionic properties, enabling the diffusion across biological membranes (Beijer and Jernelov, 1979). MeHg is lethal to plants at lower concentrations (Bizily et al., 2000), and inhibits root growth more severely than Hg(II) (Godbold, 1991). In humans, MeHg is efficiently absorbed by the digestive system (Aberg, 1969; Miettinen, 1973), and has particularly severe effects upon the nervous system. Unlike exposure to inorganic mercury, MeHg exposure tends to cause permanent symptoms, most of which are associated with the nervous system.

Extreme emotional disturbance or injury to sensory processes such as loss or distortion of vision, partial or complete deafness, and numbness in extremities or lips, are common. Also common are impairment to systems controlling muscular coordination, leading to slurred speech, difficulty with balance, and inability to control speech (D'Itri and D'Itri, 1978). A quantitative risk assessment carried out by the United States EPA suggests that the primary MeHg exposure route to piscivorious humans and animals is the consumption of fish (Keating et al., 1997). In the notorious Minimata Bay methyl-mercury poisoning episode, thousands of local humans and other animals were killed or permanently disabled as a result of the consumption of MeHg-contaminated fish (Harada, 1995).

Mercury biogeochemistry and pollution

Mercury biogeochemistry has been affected by humans to such an extent that it is impossible to separate the issue from the issue of mercury pollution. In fact, it is estimated that total global atmospheric mercury has increased by a factor of 2-5 times and the deposition of atmospheric mercury has tripled or quadrupled since the industrial revolution (Keating et al., 1997). The atmosphere is the primary medium for the global dispersal of mercury. Before industrial times, the greatest source for mercury entering the atmosphere was as Hg(0) degassing from the earth's crust (Andren and Nriagu, 1979). An anthropologically-mediated movement of mercury from the lithosphere has lead to an atmospheric load that could be as large as 5×10^9 g (Mason et al., 1994), almost all of which is Hg(0) (Matheson, 1979). Hg(0) has a mean atmospheric residence time of about one year and it is thought that 95% of the mercury that has entered the atmosphere since

1890 is now stored in soils (Keating et al., 1997). Atmospheric Hg(0) is returned to soils primarily through rainfall (Andren and Nriagu, 1979; Matheson, 1979).

Mercury in soils is typically Hg(II) in stable complexes with sulfides, organic matter or clays (Andersson, 1979) and is only desorbed very slowly (Mitra, 1986). Chemical (Albert, 1974) or microbial (Landa, 1978; Rogers, 1979) reduction of Hg(II) in soils returns Hg(0) to the atmosphere effecting the slow self-remediation of mercury pollution (Andersson, 1979). However, because of retention time of mercury in soils is long, it may be released slowly into surface waters for hundreds of years (Johansson et al., 1991) before it is naturally volatilized.

Once in marine or aquatic environments, even modest levels of inorganic mercury may be methylated to MeHg at concentrations that are hazardous to humans and/or wildlife. This process is thought to be influenced by a variety of biotic and abiotic factors though mediated most efficiently by sulfate-reducing bacteria which inhabit anoxic sediments (Compeau and Bartha, 1985). The slightly ionic nature of methylmercury allows movement to any proximal body of water where the lipophilic compound can be absorbed through the gills of aquatic or marine animals or ingested after adsorption to food particles (Windom and Kendall, 1979). After entry into the food chain, MeHg is accumulated from prey to predator in a process called biomagnification, leading to extremely high concentrations in the tissues of animals at higher trophic levels (Jernelov and Lann, 1971). When MeHg was released from an acetylaldehyde plant into Minimata Bay, Japan in the 1950s, biomagnification led to one of the most notorious environmental disasters in human history. Over 1000 people were killed and another 5000-6000 were permanently disabled (Harada, 1995; Takizawa, 1979)

The disaster may have been instrumental in exposing the dangers of environmental mercury release and in motivating governments to place tighter restrictions on mercury release. As a result, mercury has been replaced in many industrial and consumer applications (Nriagu, 1979). Today, the primary sources of environmental mercury release in the United States are fossil fuel combustion and waste incineration, together accounting for over 80% of total anthropogenic sources. Other sources are minor in comparison but are led by manufacturing industries, responsible for approximately 10% of the U.S. mercury releases (Keating et al., 1997). In developing countries, other sources of mercury release may be most significant. For example, widely-used but inefficient amalgamation mining of gold from low grade ores in the Amazon may release approximately 130-150 tons of mercury a year. This source may contribute to over 80% of the total mercury release in Brazil (Pfeiffer et al., 1993).

Mercury remediation

Most mercury at polluted sites is bound to the soil, but provides a source from which Hg(II) may dissociate and either directly affect organisms or undergo methylation and biomagnify in the food chain. Therefore, at many polluted sites, though the majority of mercury is tightly bound, the fraction that is available may be sufficient to warrant site remediation. Remediation is usually labor intensive and has typically involved extensive dredging or excavation followed by transportation to a final disposal site.

Historically, dredging of soils and sediments has been known for high cost and tendency to increase mercury mobilization into adjacent waters (Schultz et al., 1995). For example, 800 tons of wetland sediment and nearby soil were excavated from a

mercury-contaminated Superfund site in Brunswick, GA and transported to Alabama for disposal. The private portion of the funding for this project exceeded $55 million, and 380,000 pounds of mercury were unaccounted for at the end of the remediation effort (Davis, 1999). At the infamous Minimata Bay disaster area, dredging was used over a course of 10 years to move 1.5 cubic meters of contaminated sediment to a containment area located on-site (Yoshinaga, 1995). Excavated or dredged material must be relocated at considerable expense and storage conditions may lead to the conversion of inorganic mercury to MeHg (Niebla et al., 1976).

The United States EPA has stated that the BDAT (Best Demonstrated Available Technology) for mercury-containing wastes over 260 mg/kg is roasting and retorting. The method requires the transportation of soil to a roasting unit followed by a high-energy thermal processing to volatilize and then condense mercury. These wastes must then be acid-leached if it is necessary to lower the mercury concentrations below the limit of thermal recovery. Sulfide treatment of leachates from acid extractions is used to precipitate mercury for collection (Smith et al., 1995).

Because excavation and either landfilling or processing according to the EPA BDAT standard require intensive labor and energy inputs, microbial bioremediation of mercury wastes has been investigated. Studies have shown that mercury-resistant bacteria may reduce mercurial compounds from industrial waste to volatile Hg(0) (Hansen et al., 1984; Suzuki et al., 1968). However, bacteria in such studies are typically only efficient at reducing mercury in highly controlled and optimized conditions which are not representative of conditions found at mercury-polluted sites. Genetic engineering

may enable the development of microbes that are more effective in the remediation of toxic metals, including mercury (Summers, 1992).

Phytoremediation of soil metals

Plants may be more suitable than microbes for many kinds of pollution remediation. Phytoremediation is the use of plants to extract and then either detoxify or sequester contaminants from polluted soil or waters. Many authors have reviewed the potential of phytoremediation to clean or stabilize organic (Cunningham et al., 1996; Cunningham et al., 1995a; Schnoor et al., 1995) or metal (Chaney et al., 1997; Cunningham et al., 1995a; Cunningham et al., 1995b; Raskin and Ensley, 2000; Salt et al., 1995; Schnoor et al., 1995) contaminants from soil and/or water. Unlike organic contaminants, metals cannot be decomposed and therefore the most common method for soil metals "remediation" is landfilling and/or containment (Glass, 2000). Market analysis has shown that phytoremediation may have enormous economic potential as a lower cost treatment for polluted soils or waters currently cleaned by traditional methods (Glass, 1997). Landfilling together with containment is estimated to cost between $100-500 a ton whereas phytoremediation may only cost $25-$100 a ton (Glass, 2000).

In the simplest applications, plants may be used to filter contaminated waste streams or sludges in a process called rhizofiltration (Raskin and Ensley, 2000). A variety of grasses and small dicots were shown to absorb Cu^{2+}, $Cd^{2+}Cr^{6+}$, Ni^{2+}, Pb^{2+} and Zn^{2+} from hydroponic solutions (Dushenkov et al., 1995). In practical applications rhizofiltration was shown to be effective at filtering uranium from groundwater

(Dushenkov et al., 1997) and strontium from a contaminated pond near Chernobyl (Dushenkov, 1997).

In soils, the mobility of metals can vary widely depending on metal and soil characteristics (Smith et al., 1995). Phytostabilization is the use of plants to immobilize contaminants in soils, rendering them at least relatively harmless (Raskin and Ensley, 2000). Supported by aboveground productivity, plant roots can grow through many microhabitats from the soil surface to a depth of many meters and may be capable of limiting the mobility of metals throughout the area infiltrated by roots. Dense roots and canopy may reduce erosion by physically holding soil and softening rain impact, respectively. Furthermore, plants transpire significant quantities of rain water, decreasing the percolation and leaching of water through soils (Berti and Cunningham, 2000).

Because intimate root/soil contact allows plants to move soil water and nutrients to aboveground tissues, plants may be useful to clean pollutant metals from soils and then store them in aboveground tissues until later harvest and disposal. This remediation strategy is known as phytoextraction (Raskin and Ensley, 2000) or phytosequestration (Rugh et al., 2000). As many as 400 species of plants have been recognized for their capacity to accumulate high tissue concentrations of metals from soils (Kramer et al., 1997). Collectively these plants are known as hyperaccumulators. For example, species of the genus *Alyssum* may accumulate tissue concentrations exceeding 2% of plant dry weight of nickel, and species of the genus *Thlaspi* may accumulate more than 3% cadmium and 0.8% lead (Baker and Brooks, 1989). Unfortunately, most of these hyperaccumulators are of low biomass, grow slowly, or are adapted to extreme environments (Cunningham et al., 1995a). Most accumulate only one or few specific

metals, though this tendency could be considered an advantage at a site with only one metal contaminant of interest (Reeves and Baker, 2000).

Reports of successful metal phytoextraction from soil are not common in the literature. Low solubility of metals in soils may limit their absorption by roots. Furthermore, in most plants, the movement of metals from plant roots to aboveground tissues may be extremely low. Typically, most divalent cations are either chelated to organic compounds or precipitated in roots, both of which may immobilize the metal (Blaylock and Huang, 2000). This tendency may be part of a protective strategy used by plants to guard against metal toxicity in aboveground tissues. It is not entirely clear to what extent plants can sequester metals in aboveground tissues before being poisoned by the toxic effects of the metals.

Therefore, phytoextraction may not be a feasible cleanup strategy for contaminated sites unless plants are genetically engineered with enhanced capacities for metal transport and/or detoxification. Mobility from roots to shoots may be increased by the addition of chelating agents such as EDTA or EDDHA (Blaylock and Huang, 2000) to soils, however it is possible these chelating agents could cause metal to leach through soils, eventually entering groundwater. It may be more appropriate to engineer plants to excrete chelating agents or organic acids so that highest metal mobility would be in areas adjacent to roots. Roots of some grass species (Poaceae) excrete acid chelating agents called phytosiderophores to solubilize iron in soils (Ma et al., 1999). It may be possible to engineer plants to secrete ligands that are selective for other metals (Raskin, 1996).

Chelators added to a metals-spiked nutrient solution or soils may also enhance the transport of metals from plant roots to shoots (Blaylock et al., 1997). In the

hyperaccumulators of the *Alyssum* genus, plant-produced malate, citrate and histidine effectively chelate and mobilize nickel (Kramer et al., 1996). Most, if not all plants also produce polychelatins, metal-binding peptides which have been shown to be induced under heavy metal exposure (Rauser, 1995). Polychelatins escort metals such as cadmium away from proteins and organelles and into vacuoles (Salt and Wagner, 1993; Vogeli-Lange and Wagner, 1990). This suggests that plants engineered with the enhanced capacity to produce chelators (either excreted or not) may efficiently move metals to harvestable aboveground tissues, but may also have an increased tolerance to the metals.

Plants may be genetically engineered to alter the chemical state of a metal and consequently alter its mobility to aboveground plant tissues and/or the toxicity of the metal to the plant. For example, *Arabidopsis* plants have been engineered to convert arsenate to arsenite in leaves using the bacterial *ArsC* gene. Arsenate is the predominant and most root-available chemical form of arsenic in surface soils and waters. Though arsenite is more toxic, it, unlike arsenate, can be bound by metal-binding peptides inside the plant. By further engineering *ArsC* plants with the capacity to produce increased levels of metal-binding peptides, arsenate was transported to aboveground tissues where it was converted to arsenite, chelated to a harmless state and likely sequestered in cell vacuoles (Dhankher et al., 2002).

At least two metals, selenium and mercury, can be converted to volatile forms. Although plants may already carry out this process at modest levels, genetic engineering will likely be necessary to volatilize metals at a sufficiently rapid rate to clean polluted soil. This process is termed phytovolatilization (Raskin and Ensley, 2000). Plants can

concentrate selenium in aboveground tissues at different efficiencies depending on species (Banuelos et al., 1996). However selenium volatilization from contaminated soils is enhanced significantly by the presence of plants on the site (Biggar and Jayaweera, 1993). The predominant volatilized form is DMSe (dimethylselenide) of which a significant portion of the volatilized total comes either directly from plants or from soil microbes that are supported by plants. The enzymatic pathways that control plant selenium volatilization are complex but relatively well understood. Genetic engineering can be used to accelerate the rate-limiting steps in the biochemical pathway of selenium assimilation and volatilization, leading to a plant that is more efficient at phytovolatilization (de Souza et al., 2000).

Mercury volatilization from soils can be carried out chemically (Albert, 1974), and by microbes (Landa, 1978) or plants (Lindberg, 1995). However, chemical or microbial volatilization only occurs at a very shallow depth, making it unlikely that these mechanisms could move significant quantities of mercury from the soil into the air (Meagher and Rugh, 1996). Though plants can move mercury from soil and even volatilize a portion of it (Leonard et al., 1998), non-engineered plants are unlikely to volatilize mercury at rates that are useful for cleaning mercury contaminated soils. Therefore, a variety of plants have been engineered with a microbial gene, *merA,* which encodes an enzyme that catalyzes the reduction of tissue Hg(II) to volatile and less toxic Hg(0) (Heaton et al., 1998; Rugh et al., 1998; Rugh et al., 1996). Typical mercury/plant interactions and the genetic improvement of plants with the *merA* gene for mercury phytoremediation are discussed below.

Mercury interactions with plants

Like other metals, most mercury which enters plants through roots remains there (Beauford et al., 1977; Leonard et al., 1998), unless the plant is grown on extremely high levels of root-available Hg(II) (Suszcynsky and Shann, 1995). Beyond that, similarities to other metal/plant relationships are few. Plants can carry out the electrochemical reduction of background concentrations of root-absorbed Hg(II) (Hanson et al., 1995; Leonard et al., 1998), to Hg(0) which may volatilize from plants. However, it is not likely that any plants have the natural ability convert quantities of Hg(II) found at polluted sites to Hg(0) at a sufficient rate for the remediation of contaminated soils or water. Plants also carry out what is functionally the reverse process by absorbing volatile Hg(0) through stomata in leaves (Hanson et al., 1995) (Suszcynsky and Shann, 1995) where it is immobilized until leaf senescence (Lindberg, 1995). Hg(0) may be immobilized in plant leaves by conversion to Hg(II) (Gaggi et al., 1991), a process which may be facilitated by the enzyme catalase in plant cell peroxisomes (Du and Feng, 1983). Ionic mercury (Godbold, 1991) and volatilized metallic mercury (Siegel et al., 1984) are toxic to plants, though organomercurials may be substantially more toxic (Bizily et al., 1999; Godbold, 1991).

Mercury phytoremediation

Most mercury in soils may be relatively immobile. Low mercury bioavailability, while protecting plants from the toxin, may initially seem to suggest that the phytoremediation of mercury polluted soils may not be practical or necessary. However, under the appropriate conditions, even small quantities of inorganic mercury may be

methylated, then leached or washed into nearby waters where dangerous bioaccumulation may occur. In relatively dry soils, plants may be useful for the phytostabilization of mercury-polluted soils, as roots may hold mercury-covered soil particles in place and plants may transpire significant quantities of water, reducing mercury leaching.

However, the use of plants to physically remove mercury from polluted soils or sediments in an efficient manner is only likely to be feasible using genetically engineered plants. Therefore, a simple model species, *Arabidopsis thaliana*, was engineered with a modified microbial gene, *merA*, encoding a protein that catalyzes the electrochemical reduction and detoxification of Hg(II) to Hg(0) (Rugh et al., 1996). *Arabidopsis* (Rugh et al., 1996), and later *Liriodendron tulipifera* (Rugh et al., 1998), expressing this gene were highly resistant to root-absorbed Hg(II) through volatilization of Hg(0). Hydroponic assays demonstrated that *merA Nicotiana tabacum* reduce root-absorbed Hg(II) to Hg(0), removing the majority of the metal from the "belowground" system (roots and hydroponics liquid) (Heaton et al., 1998). This suggests the possibility that in soil, *merA* plants may remove Hg(II) from roots by conversion to Hg(0) which may be carried through the transpiration stream and out of the plant in an efficient phytovolatilization process. Mercury resistance of *merA* plants suggests that they may remain healthier than non-engineered plants on highly mercury-polluted soils and that their more mercury resistant root systems may penetrate contaminated soils more effectively, stabilizing soils and potentially increasing rhizospheric biological activity. Plants which typically inhabit mesic soils (*Populus deltoides*) or waterlogged sediments (*Oryza sativa*, *Juncus*, spp., *Typha* spp.) may be the most effective plants to intercept

Hg(II) before it can be converted to methylmercury and may be the best candidates for engineering with the *merA* gene.

The purpose of the following work is to investigate whether the *merA* expression in a variety of plant species causes novel plant responses to mercury exposure that are likely to enable the plants to be useful in the remediation of mercury-polluted soils. Though *Arabidopsis* and tobacco have been engineered with a bacterial gene, *merB,* which enables expressing plants to convert MeHg to Hg(II), these plants and organomercurials are for the most part beyond the scope of this study. Similarly, the idea of engineering plants to sequester mercury aboveground instead of volatilizing Hg(0) is only briefly mentioned. As the strategy for engineering plants to remediate mercury-contaminated soils develops, unique combinations of different genes may be employed to allow plants to stabilize and/or remove inorganic or organic mercury in polluted soils or waters. Mercury removed by plants will either be volatilized as Hg(0) or stored in aboveground tissues for later harvest.

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CHAPTER 2

PHYTOREMEDIATION OF MERCURY- AND METHYLMERCURY-POLLUTED SOILS USING GENETICALLY ENGINEERED PLANTS[1]

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ABSTRACT

Inorganic mercury in contaminated soils and sediments is relatively immobile, though biological and chemical processes can transform it to more toxic and bioavailable methylmercury. Methylmercury is neurotoxic to vertebrates and is biomagnified in animal tissues as it is passed from prey to predator. Traditional remediation strategies for mercury contaminated soils are expensive and site-destructive. As an alternative we propose the use of transgenic aquatic, salt marsh, and upland plants to remove available inorganic mercury and methylmercury from contaminated soils and sediments. Plants engineered with a modified bacterial mercuric reductase gene, *merA*, are capable of converting Hg(II) taken up by roots to the much less toxic Hg(0), which is volatilized from the plant. Plants engineered to express the bacterial organomercurial lyase gene, *merB,* are capable of converting methylmercury taken up by plant roots into sulfhydryl-bound Hg(II). Plants expressing both genes are capable of converting ionic mercury and methylmercury to volatile Hg(0) which is released into an enormous global atmospheric Hg(0) pool. To assess the phytoremediation capability of plants containing the *merA* gene, a variety of assays were carried out with the model plants *Arabidopsis thaliana*, and *Nicotiana tabacum* (tobacco).

Key Words: *Nicotiana tabacum*, tobacco, contaminated soil, *Arabidopsis thaliana*, phytovolatilization, phytosequestration, *mer* genes, transgenic

INTRODUCTION

Most of the mercury which has been released by anthropogenic sources is currently retained in surface soils as complexes of ionic mercury, Hg(II), bound with sulfides, clay particles or organic matter (Keating et al., 1997). The stability of Hg(II) in soils gives it an estimated mean soil residence time of at least 1000 years (Steinnes, 1995). Though the dissociation of bound Hg(II) from mineral or organic particles is limited, redistribution can occur due to bulk movement of contaminated soils (e.g. wind, surface runoff). Chemical and biological processes allow for more dynamic Hg(II) cycling via transformation to more mobile and, sometimes, more toxic species. One pathway of mercury loss from soil systems occurs through the transformation of Hg(II) to metallic mercury, Hg(0), which volatilizes from the system. This process is carried out as a mercury detoxification by some of the microbes which inhabit Hg(II)-contaminated soils. Metallic mercury is the least toxic form of mercury because of low water solubility and low reactivity (Andersson, 1979).

Inorganic mercury from anthropogenic point sources accumulates in soils and sediments where it may undergo microbial conversion to methylmercury. Methylmercury in sediments is less tightly complexed with organic and inorganic ligands than Hg(II) (Reimers et al., 1975) and is consequently more bioavailable. Furthermore, it is highly lipid soluble allowing it to be absorbed through biological membranes (Mason et al, 1996; Beijer and Jernelov, 1979). Consequently, methylmercury accumulates in aquatic or marine organisms, and efficiently biomagnifies to increasing concentrations in the tissues of animals at higher levels of the food web (Huckabee et al, 1979).

Methylmercury has been the primary toxic agent in most major environmental mercury-release disasters. The most notorious of these occurred in the 1950s when organic and inorganic forms of mercury were released from a chemical plant into Minimata Bay, Japan. Methylmercury contaminated the fish supply killing more than 1000 people and permanently injuring another 5000-6000 (Irukayama, 1977; Harada, 1995). Disasters such

as this have led to more stringent governmental regulation of mercury releases and have prompted industries to pursue alternatives to mercury use. Consequently, mercury emissions into the atmosphere have declined from their maximum rate during the 1950s and 1960s (Expert Panel, 1994). However, in some regions hazardous mercury releases have not decreased. For example, gold and silver mining in many developing nations still rely on a mercury amalgamation ore extraction process which may release large amounts of metallic mercury into aquatic, atmospheric and soil environments (Pfeiffer et al., 1993; Agaki et al, 1995). As a result of these past and current mercury releases, there are a growing number of dangerously contaminated sites that will remain environmental hazards for thousands of years unless remediated.

Mercury and plant interactions

Both organic and inorganic forms of mercury are toxic to plant roots, causing the inhibition of root elongation (Godbold, 1991) and chromosomal aberrations (Panda et al., 1992). Plants growing in Hg(II)-contaminated soil (Panda et al., 1992) or Hg(II)-spiked hydroponics medium bind and accumulate mercury almost exclusively in root tissues (Suszcynsky & Shann, 1995). Beauford et al. (1977) demonstrated that plants growing in solution culture accumulate quantities of ionic mercury in tissues at a rate that is proportional to the solution concentration of highly root-available Hg(II). Although the accumulation of mercury in different tissues of plants growing in mercury contaminated soils has been studied (Hogg et al., 1978; Lindberg et al., 1979), correlations of this mercury uptake with factors controlling mercury availability to plant roots seem to be lacking. It can be assumed that any factor that affects soil mercury mobility will influence the availability of mercury to plant roots. Soil redox potential, pH, chloride concentration, sulfur content, and percent organic matter all influence the availability of soil mercury and the microbial activities which process mercury (Adriano, 1986).

Plants can conduct mercury from soil by absorbing it through the roots and then volatilizing it from leaves as Hg(0) (Kozuchowski and Johnston, 1978). In a study of the atmosphere above a wooded area containing only background levels of mercury in soil, most Hg(0) was found to have been emitted from the plants (Lindberg, 1995). Plants may also absorb Hg(0) into leaf tissues via gas exchange with the atmosphere (Hanson et al., 1995). Once inside the leaf, Hg(0) is enzymatically oxidized to Hg(II) which is retained until the return of plant tissues to the soil via litterfall (Lindberg, 1995). Hg(0) vapor which is accumulated as Hg(II) in aboveground tissues is not efficiently transported to roots (Lindberg et al., 1979; Suszcynsky and Shann, 1995).

Plant potential for mercury phytoremediation

Mercury contaminated soils and sediments are commonly remediated by soil excavation, relocation and burial. This process is costly, physically degrades the site and may cause extensive loss of mercury from the site during the remediation effort. Phytoremediation could cost-effectively replace traditional mercury remediation strategies. Plants extract metals from soil while minimizing soil erosion, enhancing soil structure, promoting subterranean biological activity, and reducing soil concentrations of pollutants such as phosphate, nitrate, or even toxic organics. However, the use of phytoremediation to replace traditional mercury remediation strategies faces several challenges. The availability of soil mercury to plant roots may be one of the rate limits to the phytoremediation process. Furthermore, the inability of mercury to travel from plant roots to aerial tissues suggests that only plants that are genetically engineered to mobilize mercury will have the ability to transport substantial quantities of mercury out of the soil/root system.

Microbes that inhabit mercury-contaminated environments have evolved mercury resistance which is genetically encoded by the *mer* operon (Summers, 1986). Within this operon, two genes, *merA* and *merB*, code for catalytic mercury-processing enzymes. *MerB* encodes organomercurial lyase that catalyzes the cleaving of the carbon-mercury bond in a

variety of organomercurial compounds, such as methylmercury (MeHg) and phenylmercuric acetate (PMA). The metal product of this reaction, Hg(II), is the substrate for the enzyme mercuric ion reductase, encoded by *merA*. *MerA* catalyzes the electrochemical reduction of this Hg(II) to Hg(0). These reactions lower the relative toxicity of the metal and allow it to be volatilized from the system. Mercury-resistant bacteria have been considered for bioremediation of mercury wastes, though they appear to be effective only under narrow, controlled conditions and are probably not applicable for use in a field environment (Suzuki et al., 1968, Hansen et al., 1984).

We propose the remediation of mercury contaminated soils and sediments using plants that have been genetically engineered with genes from mercury-detoxifying bacteria. These plants would extract Hg(II) and methylmercury from contaminated soils or sediments and then convert the mercury forms to Hg(0) which would volatilize from the plants and/or be sequestered in shoot tissues as Hg(II) (Fig. 2.1). Because methylmercury is a greater problem in aquatic and marine sediments than in terrestrial soils, most mercury phytoremediation will likely be carried out with aquatic and salt marsh plants. Therefore, we are currently working to introduce the *merA* and *merB* genes into the salt marsh plant *Spartina alterniflora* (cordgrass), the semi-aquatic plant *Oryza sativa* (rice) and aquatic emergents *Juncus spp*. and *Typha latifolia* (cattail). We aim to further genetically engineer *merA*/*merB* plants so that a substantial portion of processed mercury is stored in aboveground tissues for later harvest and transport to a disposal area. Plants of this type could be used as an alternative to Hg(0)-volatilizing plants in circumstances where the release of Hg(0) into the regional environment might be ill-advised.

MERCURY PHYTOREMEDIATION

Development of transgenic plants for mercury resistance

Standard techniques for plant genetic engineering were used to transfer the bacterial *merA* and *merB* genes into *Arabidopsis thaliana* L. (a small, weedy member of the Mustard family) (Rugh et al., 1996) and *Nicotiana tabacum* L. (tobacco). These two plant species were chosen to test the utility of *mer* gene constructs because they are easily genetically manipulated and have relatively short life cycles, traits that make them commonly used research tools. *MerA-Arabidopsis* and *merA*-tobacco seeds germinated and grew vigorously on substrates containing 10 ppm Hg(II), a concentration toxic to seeds of the non-transformed wild-type. The *merA* plants appeared healthy with full, green leaves, branching roots, and normal flower and seed formation.

MerB-Arabidopsis plants proliferate on growth medium containing concentrations of phenylmercuric acetate (PMA, ~300 ppb) that are lethal to non-transgenic controls (Bizily et al., 1998). PMA causes mortality in wild-type *Arabidopsis* plants at lower concentrations than Hg(II) [10,000 ppb versus 300 ppb, respectively]. The basis for survival of *merA*-plants on Hg(II)-spiked medium is the enzymatic reduction of highly toxic and reactive Hg(II) to relatively benign Hg(0), which rapidly evaporates from the tissues and medium. However, *merB*-plants grow on elevated levels of organic mercury by creating Hg(II) as a product of their reaction, though in small amounts relative to the levels of Hg(II) that are toxic for wild-type plants. A plant engineered with both *merA* and *merB* would couple these two processes to convert both methylmercury and Hg(II) to Hg(0).

Mercury phytovolatilization

Aqueous solution medium was used to maximize the bioavailability of Hg(II) for plant uptake and Hg(0) volatilization assays. The ability of *merA-Arabidopsis* plants to convert Hg(II) in buffered reaction medium to Hg(0) vapor was measured using closed-tube

incubation assays (Rugh et al., 1996). Plants were placed in a test-tube containing 1.5 ml of 5 ppm Hg(II) solution and sampled by sparge-evacuation of the headspace each minute over the course of a ten minute assay. The samples were collected and measured using a Jerome 431 mercury vapor analyzer (Arizona Instruments, Phoenix, Arizona, USA). The transgenic plants volatilized 3-4 times more Hg(0) per mg of plant tissue than wild-type controls. These data confirmed the mechanism of resistance by *merA*-plants to be conversion of Hg(II) by the MerA enzyme and the consequent release of relatively inert Hg(0).

Tobacco hydroponics assays were used to measure plant root uptake and reduction of solution Hg(II). Earlier work performed upon non-transgenic tobacco showed that only at extremely high media concentrations (~100 ppm Hg(II)) does the shoot begin to accumulate greater than 10% of the total plant load (Suszcynsky and Shann, 1995). Experiments were conducted with Hg(II) concentrations that were reported to cause no apparent long-term plant stress. This allowed a clear evaluation of the effects conferred upon transgenic plants by the expression of the MerA enzyme, without exposing control plants to acutely toxic Hg(II) concentrations. Tobacco plants were grown in Fafard 3B potting soil (Conrad Fafard, Inc., Agawam, Massachusetts, USA) to approximately 10 inches of height and then transferred, with extensive root washing, into hydroponic containers (~1000 ml nutrient medium in Ball canning jar) (Alltrista Corp., Muncie Indiana, USA). Plants were allowed to acclimatize to the aerated, hydroponics environment for 7 days. After this adjustment period, fresh media was substituted and spiked to 1 ppm Hg(II). Wild-type and *merA* plant roots bound >99% of the Hg(II) from the medium within 24 hours of addition. After 1, 3 and 7 days, roots of each genotype were harvested, freeze-dried and digested for mercury analysis as described in Suszcynsky and Shann (1995). Each digest was analyzed for total mercury using cold vapor atomic absorption spectrophotometry (Thermo Jarrell-Ash SH1000, UGA Chemical Analysis Laboratory).

After 3 days, significantly less mercury was found in the roots of two different *merA* lines than in wild-type roots ($p = 0.0014$ and $p = 0.0096$, respectively) (Fig. 2.2).

The trend continued through the day 7 harvest by which time *merA* plants had removed more than 70% of the total system Hg(II). In contrast, by day 7 wild-type plants had removed less than 20%. Hg(II) lost from the *merA* roots was assumed to have been removed from medium and reduced to less toxic, volatile Hg(0) which was emitted from the plant tissues.

MerA tobacco on ionic mercury-spiked soil

Hg(II)-spiked hydroponics provide only a limited simulation of soil systems because mercury in Hg(II)-contaminated soils is far less available to roots. Hg(II)-spiked soils were used to assay biomass accumulation and mercury uptake in *merA* and wild-type tobacco. These assays provide the first simulations of the phytoremediation capacity of *merA* plants. An unsieved commercial potting soil (Fafard 3B) was spiked with Hg(II) for use in plant growth and survival analyses. The potting soil was rich in organic matter (67% weight lost at ignition), making it dissimilar to most field soils. However, the soil was ideal to demonstrate that even in soils where Hg(II) is highly bound to organic matter, there is enough root-available Hg(II) to easily differentiate between *merA* and wild-type plant response. For all but the control soils (0 ppm Hg(II)), dissolved $HgCl_2$ was mixed into the soil to a final concentration of either 100 or 500 ppm Hg(II) at 60% of water holding capacity. Wild-type and *merA*-tobacco seeds were plated and germinated on agar-gel growth medium to select for uniform germinant size and vigor. Each selected plantlet (~1 cm tall) was transferred to soil in a 2.5 x 2.5 inch plastic pot, set within a petri-dish bottom and arrayed on a platform table in a completely randomized 2-way ANOVA design. The two treatment factors were soil Hg(II) concentration (0, 100, 500 ppm), and tobacco genotype (wt, *merA*). Five replicate plants for each treatment combination were grown in growth chambers at 25°C with alternating 16 and 8 hour periods of light and darkness, respectively. Bottom-watering of the plants was performed by adding an equal volume of tap water to each petri-dish every 48 hours.

The first apparent evidence of Hg(II) toxicity to the wild-type plants was reduced size on Hg(II)-spiked soils, unlike the *merA*-tobacco plantlets which were of similar size in all treatments (Fig. 2.3). All wild-type and *merA* plants survived in this experiment, with the exception of one wild-type plant which died in the 500 ppm Hg(II) soil. After 4 weeks of growth, the aboveground plant tissues were harvested and weighed. The shoot biomass of surviving wild-type and *merA* tobacco plants were measured as an indication of capacity to resist soil Hg(II). The shoot fresh weight of *merA* plants was significantly greater than that of wild-type control plants in all treatments (t-test; $p = 0.02$ for 0 ppm, $p = 0.0002$ for 100 ppm, and $p = 0.0014$ for 500 ppm) (Fig. 2.4A). It was expected that shoot fresh weight of *merA* plants would exceed that of controls in both Hg(II)-containing soils. However, even in soils with 0 ppm Hg(II), *merA* plants had significantly greater fresh weight than controls. These controls may have been stressed as a result of their foliar uptake of Hg(0) that had been released from adjacent soils and plants.

The plant shoots were prepared for tissue mercury analysis using the same methods applied to the hydroponically grown plants. In the 0 ppm and 500 ppm Hg(II) soil, mercury concentrations in *merA* shoots were significantly lower than in aboveground wild-type tissues (t-test; $p < 0.0001$ and $p = 0.0005$, respectively) (Fig. 2.4B). In the 100 ppm Hg(II) soil the trend was similar, however the difference was not statistically significant ($p = 0.1319$). The total shoot mercury loads for wild-type and *merA* plants in mercury-spiked soils did not differ significantly (Fig. 2.4C). However, in the 0 ppm Hg(II) treatment, total shoot mercury load was significantly higher in wild-type plants than *merA* plants ($p < 0.0001$). This finding supported the supposition that control plants in 0 ppm Hg(II) soil absorbed atmospheric Hg(0) in their leaves, accounting for at least some of the difference in shoot fresh weights between wild-type and *merA* plants. Although there were strongly significant differences between wild-type and transgenic plants in shoot Hg(II) concentration, there was not a compelling difference between the genotypes in total mercury load. Although it may be expected that *merA* plants would have a lower tissue mercury load

than wild-type plants, *merA* plants also had greater tissue mass in which to store Hg(II) on tissue surfaces, in cell walls or other inactive sites. MerA activity allowed the transgenic plants to grow with little or no growth inhibition suggesting that the mercury load contained in the plants had not greatly accumulated within vital cells and tissues.

DISCUSSION

Data summary

MerA-engineered plants show promise as tools for mercury phytoremediation. They survive on concentrations of Hg(II) in growth media that are toxic to wild-type plants and remove significantly more mercury from a spiked hydroponics system than wild-type plants. These observations suggest that *merA* plants can efficiently transform root-available Hg(II) to the less toxic Hg(0). On mercury-spiked, high-organic matter soils, enough root-available Hg(II) is present to cause significantly slower growth in wild-type than *merA*-plants. This indicates that the transgenic plants are able to process the mercury to detoxified and removable Hg(0). At contaminated sites, this bioavailable Hg(II) is the pool from which methylmercury is produced. Therefore, the use of *merA*-plants to process mercury from this pool will slow the production of methylmercury in soils and sediments. Plants expressing the *merB* gene could offer an additional level of protection by removing and breaking down methylmercury before it can accumulate. Plants expressing both *merA* and *merB* genes would be capable of converting both Hg(II) and methylmercury to volatile Hg(0). The demonstration that plants can be engineered with bacterial genes for mercury detoxification suggests that it may be possible to install plant communities for economically feasible and environmentally-compatible mercury pollution remediation.

Consideration of phytovolatilization versus phytosequestration

Using plants to accelerate the volatilization of Hg(0) from mercury-contaminated sites may in many cases be the most feasible remediation option. Volatilization is a permanent site solution as the Hg(0) removed is not likely to be redeposited at or even near the site. Furthermore, plant-assisted Hg(0) volatilization may require little site management after the original planting effort. Site disturbance would be limited to plantings minimizing site erosion and consequently minimizing the transport of colloid- and organically-bound mercury off-site. In practice, phytovolatilization would release an amount of Hg(0) into the atmosphere that would not substantially add to the enormous pool that currently exists. However, accelerating the emission of Hg(0) into a regional atmosphere could be unwise under certain conditions. The direction of prevailing winds, the location of population centers, and the magnitude of site Hg(0) emission would all play vital roles in determining whether Hg(0) release could pose a hazard to humans or ecosystems.

If the safety of using phytovolatilization at a site were questionable, the site could be remediated using plants that sequester mercury in aboveground tissues. Plants do not normally transport or accumulate greater than trace amounts of mercury in their shoot tissues. Plants used for mercury phytosequestration in the field would be genetically engineered with the *merA* and *merB* genes in roots only and with an increased enzymatic capability to oxidize root-generated Hg(0) to bound Hg(II) in aboveground tissues (Fig. 2.1C). The shoots would be harvested after plants had achieved their maximum growth, processed by dehydration to minimize mass and volume, and disposed of as hazardous waste. Phytosequestration would reduce Hg(0) volatilization from a site, but would require substantial site maintenance for planting and harvesting. These requirements combined with transport and waste processing fees would incur higher costs than treatment by phytovolatilization, but likely far less than bulk soil excavation and burial. Furthermore, phytosequestering plants may allow for mercury ingestion by herbivores during the field growing stages of the cleanup operation. "Clean crops" could be planted adjacent to the

remediation site to attract animals from Hg(II)-loaded plants. This would have to be supplemented by more aggressive deterrents, such as fences and repellents.

Application of genetic engineering to phytoremediation

Our research has indicated the potential utility of using genetic engineering to develop plants with exceptional capabilities for soil mercury detoxification and removal. Applied plant modification has existed nearly as long as agriculture itself. Today, explosive advances in crop improvement have employed molecular genetic approaches to enhance crop quality, productivity and resistance to a variety of biotic and abiotic stresses. We have begun to apply these powerful tools to problems of soil mercury contamination. Plants have evolved complex mechanisms to carry out an amazing range of biochemical and physiological processes. Plant genetic manipulation will allow us to both optimize natural plant processes and further supplement them with novel traits from organisms outside the plant kingdom. For example, if *mer*-plants were unable to grow successfully on sites contaminated with other phytotoxic pollutants, their tolerance to these toxins could be enhanced by additional genetic engineering. Ongoing and future research will allow the introduction of single genes and potentially even complex traits for the detoxification of a variety of hazardous materials, such as organic compounds or heavy metals other than mercury.

The use and release of genetically engineered organisms into the environment has been intensely scrutinized from a wide spectrum of disciplines. The most common concern is that the transgenic organisms will migrate outside of their intended range and displace natural species. Unquestionably, care must be taken to prevent the release of harmful engineered organisms such as "weedy" plants. However, extensive laboratory, growth chamber, and controlled field experiments are always performed, giving a clear understanding of the characteristics of improved organisms. The introduction of a novel gene into a species can allow for its easy tracking and identification, so that if environmental removal of the plant becomes necessary, it may be feasible. There may be self-regulation of

plant dispersal in plants engineered for detoxification and/or storage of toxic substances. In the absence of the hazard, the specialized trait could become more of a cost than an ecological advantage and the plant may fail to out-compete natural, wild-type plants. Most importantly, the value gained by genetically-designing highly specialized plants for removal of dangerous hazards greatly substantiates research toward their development.

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Figure 2.1. Illustration of Hg-interaction with: **A.** wild-type, **B.** whole-plant *merA* and *merB* (phytovolatilization), and **C.** root-only *merA* and *merB* (phytosequestration) plants.

Figure 2.1

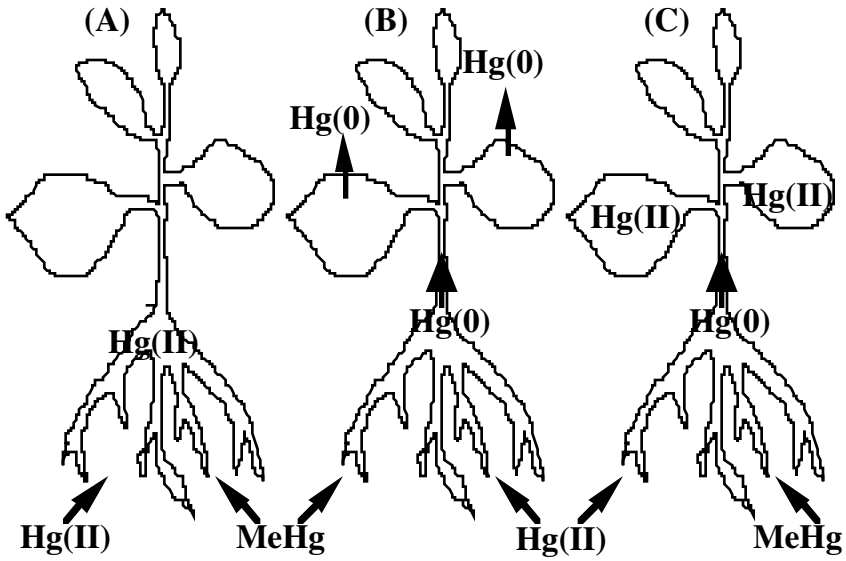


Figure 2.2 Tobacco-hydroponic assay: total mg of Hg(II) remaining in plant tissues of wild-type and merA-15.2 and merA-2.1 tobacco at each sample day. Errors are expressed as one standard error of the mean.

Figure 2.2

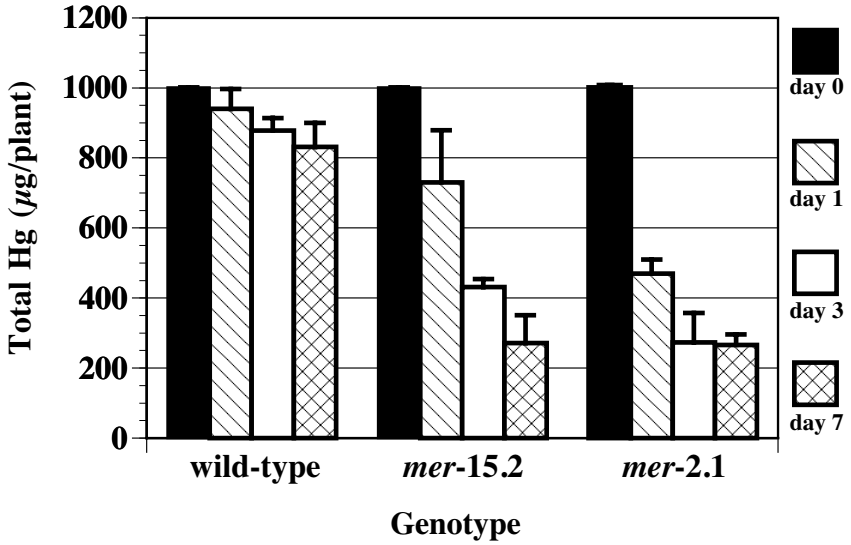

1200
1000
800
600
400
200
0
Total Hg (μg/plant)
wild-type
mer-15.2
mer-2.1
Genotype
day 0
day 1
day 3
day 7

Figure 2.3. Wild-type and transgenic *merA*-expressing tobacco plants growing on three Hg(II) concentrations (ppm) in potting soils.

Figure 2.3

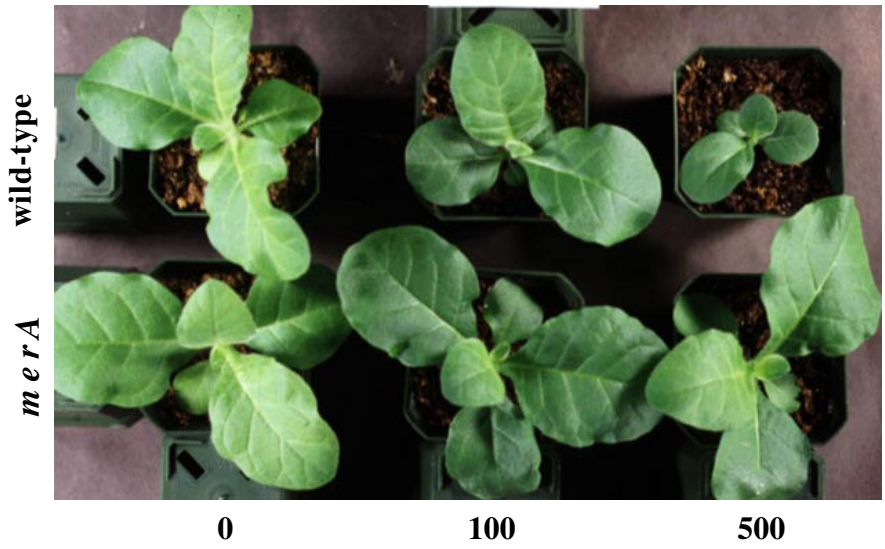
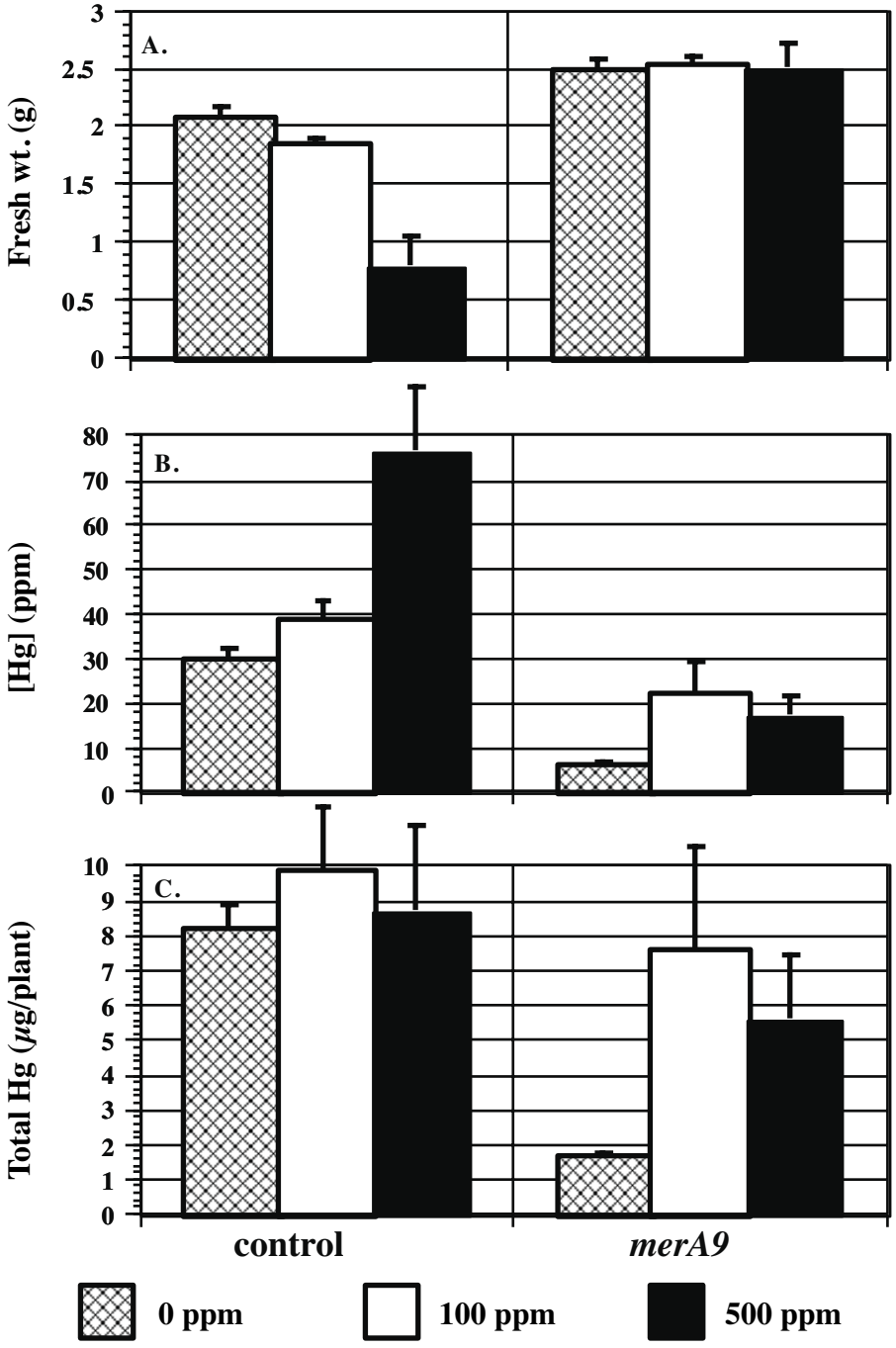


Figure 2.4. A. Plant shoot fresh weight (g), B. Hg concentration (mg/g) in shoots, and C. total Hg in shoots (mg/plant) of tobacco plants grown in Hg-amended soil. Control plants are wild-type.

Figure 2.4


A.
Fresh wt. (g)
3
2.5
2
1.5
1
0.5
0
B.
[Hg] (ppm)
80
70
60
50
40
30
20
10
0
C.
Total Hg (μg/plant)
10
9
8
7
6
5
4
3
2
1
0
control
merA9
0 ppm
100 ppm
500 ppm

CHAPTER 3

MERA- ENGINEERED TOBACCO AS A MODEL FOR MERCURY PHYTOREMEDIATION[1]

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[1]Heaton, A.C.P., C.L. Rugh, N.J. Wang, and R.B. Meagher. To be submitted to *Plant Physiology*.

ABSTRACT

Plants expressing a modified bacterial mercury reductase, *merA*, are highly resistant to Hg(II) toxicity as a result of the enzymatically-catalyzed conversion of Hg(II) to the much less toxic and volatile Hg(0). MerA expression may enhance Hg(II)-reductive capacity and mercury resistance to the extent that *merA* plants exhibit a suite of responses to mercury which make them more capable than wild-type plants of interacting with and removing mercury from soil or water. We have engineered *merA* expressing *Nicotiana tabacum* (tobacco) as a model to examine these processes. Mercury resistance was demonstrated by germinating and growing healthy *merA* tobacco on semi-solid medium spiked with a $HgCl_2$ concentration acutely toxic to the wild-type. On similar medium, *merA* plant roots penetrate highly concentrated, localized Hg(II) hot-zones composed of HgS (cinnibar) more effectively than the wild-type. In hydroponic medium, *merA* plants suffered significantly less reduction of transpiration than the wild-type when medium was spiked with $HgCl_2$. The ability of *merA* Hg(II)-reductive activity to counter typical plant-catalyzed Hg(0) oxidation to Hg(II) was demonstrated by a lower net absorption of atmospheric Hg(0) than wild-type. The role of *merA* in altering the mobility of mercury within plants was examined by reciprocally grafting *merA* and wild-type tobacco at the root/shoot junction and growing plants on $HgCl_2$-spiked hydroponic medium. Elevated mercury concentrations in wild-type shoots grafted to *merA* roots suggest the movement of mercury from *merA* roots to wild-type shoot possibly by repeated electrochemical oxidation and reduction of Hg(0) and Hg(II), respectively. Understanding *merA*-facilitated changes to normal plant/mercury interactions should guide further plant improvement for efficient phytoremediation.

INTRODUCTION

Plants have been suggested as tools for environmental pollution remediation. The use of plants to absorb and then either detoxify or sequester contaminants from water, air or soil is termed phytoremediation. Initial investigations using phytoremediation to clean organic pollutants from soil or water have been relatively successful because native or engineered plants can often convert complex organic toxins to harmless small molecules (Cunningham and Ow, 1996; Meagher, 2001). However, phytoremediation of metal pollution in soils faces greater challenges because toxic metals cannot be degraded to benign components and are thus usually excluded from plant roots or are sequestered at relatively low concentrations in root tissues (Cunningham et al., 1995). Mercury is unique as a metal because it can be completely reduced to an uncharged, less toxic, and volatile metallic form, Hg(0). Plants can carry out the electrochemical reduction of background levels of root-absorbed Hg(II) (Hanson et al., 1995; Leonard et al., 1998), but are not likely to have the natural ability convert Hg(II) to Hg(0) at a sufficient rate for efficient remediation of highly contaminated sites.

The bacterial *merA* gene encodes an enzyme which catalyzes the reduction of ionic mercury, Hg(II), to the less toxic and volatile metallic mercury, Hg(0) (Fox and Walsh, 1982). However this gene has a highly skewed, GC-rich codon composition making it unsuitable for strong and stable plant expression. For efficient plant expression that is stable for many generations, directed sequence mutagenesis was used to create the *merA9* gene from the original bacterial *merA* gene. *merA9* was introduced into *Arabidopsis* (Rugh et al., 1996), *Liriodendron tulipifera* (Rugh et al., 1998), *Nicotiana*

tabacum (Heaton et al., 1998), *Oryza sativa* (Heaton et al., submitted), and *Populus deltoides* (Che et al., submitted) with the goal of engineering a mercury phytoremediation system for soil and/or water. In a similar manner, the *merA9* gene was further modified to yield *merA18* which was introduced into *L. tulipifera* (Rugh et al., 1998). Plants expressing *merA9* or *merA18* are highly resistant to toxic Hg(II) in growth substrates via reduction to the much less toxic Hg(0), most of which volatilizes from the plant (Rugh et al., 1996; Rugh et al., 1998).

Because of moderate size, rapid growth and relatively short life cycle, tobacco is an ideal model species for studying the physiological effects of *merA*-mediated Hg(II) reduction. Mature *merA9* and wild-type tobacco were grown on $HgCl_2$-spiked hydroponics medium for 7 days and were shown to remove virtually all of the Hg(II) from the medium (Heaton et al., 1998). *merA* plants converted 70% of the extracted Hg(II) to Hg(0) which was volatilized. In contrast, less than 20% of the mercury was removed by wild-type tobacco and virtually all of the remainder was found bound to the roots at the end of the assay. This is a typical result as most root-absorbed Hg(II) remains in roots of non-engineered plants (Beauford et al., 1977; Leonard et al., 1998).

Hg(II) has a significant and detrimental affect on the roots of non-engineered plants. Root uptake of Hg(II) may reduce water flow in roots by inhibiting root metabolism and consequently slowing the activity of water channel proteins called aquaporins (Zhang and Tyerman, 1999). The direct reaction of Hg(II) with sulfhydryl groups on aquaporins may lead to physical blockage and may further contribute to transpiration reductions (Maggio and Joly, 1995). Furthermore, Hg(II) may reduce root

elongation through root damage (Godbold, 1991), or in soils may detrimentally affect the diameter of root/rhizome size (Cocking et al., 1995).

Plants have been shown to absorb volatile Hg(0) through stomata (Hanson et al., 1995; Suszcynsky and Shann, 1995), converting it to an immobile form in leaves, where most of it remains until leaf senescence (Lindberg, 1995). It has been suggested that Hg(0) is immobilized in plant leaves by oxidation to Hg(II) (Gaggi et al., 1991), a process which may be facilitated by the enzyme catalase in plant cell peroxisomes (Du and Fang, 1983). A significant concentration of mercury was found in leaves of wild-type and *merA* tobacco plants grown on clean soil but adjacent to $HgCl_2$-spiked soil/plant systems which may have volatilized Hg(0) (Heaton et al., 1998). Though the source was not experimentally controlled, aboveground tissue concentrations indicated that wild-type plants absorbed or retained more of this Hg(0) than *merA* plants. This is consistent with the idea that activity of the *merA* gene in converting Hg(II) to Hg(0) is the reverse of a significant plant-mediated Hg(0) immobilization through oxidation to Hg(II). If true, then both mercury oxidation and reduction reactions may be present in tissues of *merA* plants, possibly leading to repeated reduction and oxidation of mercury within plants. As Hg(0) is water-insoluble but non-reactive and Hg(II) is highly water soluble but highly reactive, the potential ability of *merA* plants to transport mercury through tissues is difficult to predict.

The simple biochemical conversion of Hg(II) to Hg(0) will not enable phytoremediation to succeed unless this reaction leads to other advantages in function of plants growing on mercury. Therefore a series of experiments has been conducted in order to assess the impact that the *merA* gene has on plant mercury resistance, uptake and

processing in tobacco. *merA9, merA18* and wild-type tobacco were exposed to mercury in a variety of media to demonstrate the connection between mercury resistance and Hg(II)-reduction, both of which are enhanced in the *merA* genotypes compared to the wild-type. *merA* Hg(II) resistance was demonstrated through exposure of plants to levels of $HgCl_2$ in semi-solid growth medium normally acutely toxic to the wild-type. Differences between wild-type and *merA* root growth and plant transpiration were demonstrated by exposure of plant roots to sub-lethal levels of Hg(II). Finally, exposure of leaves to Hg(0) and roots to highly bioavailable Hg(II) was used to contrast the ability of *merA* tissues to reduce Hg(II) to Hg(0) with the natural plant tendency to immobilize Hg(0), possibly as Hg(II).

RESULTS

Transformation verification and germination on toxic level Hg(II)

Stable transformation of tobacco with *merA9* and *merA18* genes was confirmed using PCR amplification of a small fragment of each *merA* construct, using DNA prepared from T_1 generation plants (Fig. 3.1A). The majority of plant lines surviving on kanamycin/timentin plates contained the *merA* gene. Western blots confirmed protein expression in PCR-positive transformants by reaction of MerA-specific antibody with the expected 60 kD protein (Fig. 3.1B). Kanamycin resistant plants had sufficient MerA protein expression that it was easily detected. Seeds of plants engineered with either the *merA18* and *merA9* genes germinated and grew well on Murashige and Skoog (MS) medium containing 60 μM $HgCl_2$ (Fig. 3.2). The wild-type controls did not germinate on

similar mercury-spiked growth medium. Results are consistent with earlier germination assays which showed *merA* expression in tobacco can be confirmed reliably by screening seeds on semisolid MS medium spiked with at least 50 μM $HgCl_2$ (Heaton et al., 1998).

Enhanced penetration of HgS obstacle by *merA* roots

The high Hg(II) resistance of *merA* plants suggested that *merA* and wild-type tobacco roots might differ in their abilities to penetrate soil or media with regions of extremely high, but localized Hg(II). In order to mimic a soil heterogeneously contaminated with mercury while allowing root growth to be easily observed, the experiment was carried out on semi-solid MS growth medium. A mercury compound was desired which would not dissolve readily in this medium, but which would have substantial availability to roots upon direct contact. When plates containing wild-type and *merA* tobacco on MS semi-solid growth medium were implanted with HgO (Ksp = 3.6×10^{-26}), the wild-type died within 1 week and *merA* plants survived indefinitely (Fig. 3.3A&B). This indicates a significant dissociation and diffusion of Hg^{2+} from HgO. When a similar assay was carried out with HgS (Ksp = 2×10^{-54}) instead of HgO, both the *merA* and the wildtype survived with no visible initial growth difference. This indicates that as predicted by solubility product constants, Hg^{2+} dissociates in semi-solid MS growth medium much more readily from HgO than HgS.

Wild-type *merA18* and *merA9* plants were grown vertically with roots moving downward toward a groove lined with HgS (Fig. 3.3C&D). The groove extended across the plate and penetrated to the bottom of the plate, ensuring roots would encounter the HgS. Significantly more *merA* than wild-type roots crossed this groove (Fig. 3.4)

(*merA18*: n = 2, p = 0.072, *merA9*: n = 2, p = 0.095, *merA*tot: n = 4, p = 0.002). Both wild-type and *merA* plants appeared healthy for the duration of this assay.

Effect of root-absorbed $HgCl_2$ on transpiration

Wild-type and *merA18* tobacco were compared in their ability to transpire water when grown on dilute hydroponics medium. Transpiration rates were assayed before and after the addition of a $HgCl_2$ spike that was calculated to bring the medium to a sub-lethal concentration of 25 μM Hg(II). Transpiration, expressed as a percentage of water loss from hydroponics jars over 48 h compared to loss during a 48 h period immediately before day 0, was statistically identical for the two genotypes before the $HgCl_2$ spike. Initial transpiration volume was approximately 140 ml·plant^{-1} for the 48 h period immediately before day 0, averaged over both genotypes. The mercury spike was added immediately after the day 4 water replenishment and water loss measurement. Transpiration percentage of original was significantly lower for the wild-type than for the *merA* at all time points after the mercury spike (Fig. 3.5) (day 6: p = 0.03, day 8: p = 0.02, day 10: p = 0.02). Forty-eight hours after the mercury spike (day 6), transpiration in wild-type tobacco decreased to approximately 25% of the pre-spike volume whereas the *merA* plant transpiration volume only decreased to 50%. Similar differences between wild-type and *merA* plants were observed on day 8 and 10.

Hg(0) oxidative capacity measured as Hg(0) absorption from air

The difference in absorption and retention of atmospheric Hg(0) by wild-type and *merA* tobacco plants was compared by incubation of plants for 5 h in a chamber

containing elevated air Hg(0) concentrations. Temperature in the semi-closed chamber was 29.5˚C when plants were first enclosed. As expected, temperature in the chamber increased at an even rate, reaching 32˚C by the end of the assay (h = 5). Hg(0) concentration in the chamber increased from 0 to 54.3±1.4 $\mu g \cdot m^{-3}$ in the first hour and only gradually increased for the duration of the assay to a final and maximum concentration of 74.8±6.8 $\mu g \cdot m^{-3}$.

The concentration of mercury in tissues of *merA* tobacco harvested immediately after the incubation was significantly lower than in wildtype tissues (t-test p = 0.003), indicating a higher net Hg(0) absorption by wild-type than by *merA* plants (Fig. 3.6). Wild-type and *merA* tissue mercury concentrations were 35.71 ±4.50 and 4.35±0.83 $\mu g \cdot g$ plant material $(dw)^{-1}$, respectively. Plants allowed to remain in clean air for an additional 24 h after the 5 h incubation (identified as "+24 h" plants) contained less mercury in tissues than plants harvested immediately after the incubation, indicating a loss of mercury (Probably as volatile Hg(0)) during the 24 h period. Surprisingly, this tissue mercury concentration difference between the 5 h and +24 h plants was only significant for the wild-type (t-test, p = 0.056). However, the +24 h wild-type contained a mercury concentration almost 6 times that of the +24 h *merA*. The difference between +24 h wild-type and +24 h *merA* mean tissue mercury concentrations was highly significant (t-test, p = 0.004).

Mercury transport through and retention in tissues: reduction vs. oxidation

An assay was designed to assess whether Hg(II) can be converted to Hg(0) in *merA* roots and then moved vertically through the plant. Wild-type and *merA18* tobacco

were grafted reciprocally (*merA* root grafted to wild-type shoot and reverse), or in a same-genotype manner (*merA* or wild-type root regrafted to *merA* or wild-type shoot, respectively), and were grown for 10 days in growth medium spiked with 5 μM $HgCl_2$. Preliminary hydroponic assays showed that mercury concentrations in root, leaf and stem of same-genotype grafted tobacco plants were statistically identical to ungrafted plants of the same genotype (data not shown). Graft unions did not alter movement or processing of mercury in plants and therefore ungrafted controls were not included in subsequent assays. As has been seen in earlier hydroponic studies (Heaton et al., 1998), mercury remaining in the medium by the end of the assay was less than 1% of the initial spike for all genotypes. Also similarly to these earlier studies, *merA* roots had significantly lower mercury concentrations than wild-type roots by the end of the study, indicating that mercury was likely removed from tissues by Hg(II)-reductive activity of the *merA* gene (Fig. 3.7).

Leaf tissues of the $wt_s/merA_r$ (wild-type shoot / *merA* root) genotype combination were significantly higher in mercury concentration than all other genotype graft combinations (vs. wt/wt $p = 0.15$, vs. $merA_s/merA_r$ $p = 0.09$, vs. $merA_s/wt_r$ $p = 0.06$), which were all statistically identical to each other (Fig. 3.7). The $wt_s/merA_r$ plants also had the highest concentration of mercury in stems, with high statistical significance compared to the two other genotype combinations with wild-type roots (vs. wt_s/wt_r $p = 0.0001$, vs. $merA_s/wt_r$ $p = 0.0002$), but less statistical significance compared to the $merA_s/merA_r$ plants ($p = 0.05$). The $merA_s/merA_r$ stem tissues themselves had higher mercury concentrations than stems of plants with wild-type roots though statistical significance was more modest (vs. wt_s/wt_r $p = 0.14$, vs. $merA_s/wt_r$ $p = 0.19$). Mercury

concentrations in aboveground tissues were not of a sufficient magnitude to account for the large differences between *merA* and wild-type root concentrations. This demonstrates that volatile loss of Hg(0), not aboveground sequestration was the primary fate of mercury mobilized by *merA* roots.

DISCUSSION

As seen in previous studies, the *merA* gene confers plant resistance to Hg(II). Resistance is demonstrated by the vigorous growth of *merA* tobacco on semi-solid growth medium spiked with 60 μM Hg(II), a concentration of sufficient toxicity to prevent wild-type seed germination. However, *merA*-catalyzed Hg(II) reduction has more complex effects on expressing plants exposed to mercury than simply allowing survival on normally acutely toxic concentrations of root-available Hg(II). The enhanced ability of *merA* roots to cross a barrier of highly concentrated HgS demonstrates a difference between *merA* and wild-type root response to Hg(II) toxicity. Typically, exposure to mercury inhibits root elongation through root damage (Godbold, 1991) and in field soils may have an inverse relationship with root or rhizome diameter (Cocking et al., 1995). Perhaps as the plants extract nutrients such as zinc or magnesium from the medium, they concomitantly extract mercury from the HgS complex. *merA* roots remain uninhibited by Hg(II) and grow efficiently through the HgS barrier.

The results of this experiment have positive implications for the use of *merA* plants in mercury phytoremediation. One common criticism of phytoremediation is that

plants may flourish on soils contaminated with high and heterogeneous concentrations of toxic pollutants, but that roots will tend to avoid penetration of contaminant "hotspots." This may result from a tendency of typical (wild-type) plant roots to grow away from the highest concentrations of mercury into relatively clean soil. However, roots of *merA* plants, suffering less stress from mercury, may grow according to other more dominant cues such as moisture, gravity and available nutrient levels. Consequently, as *merA* plants grow through localized regions of mercury contamination, they may loosen Hg(II) from the soil surface using acidic exudates normally secreted to extract nutrient ions. After mobilizing the ion, plants may directly draw Hg(II) into roots from the affected soils, and may stimulate microbial activity in the rhizosphere. Enhanced microbial activity in the rhizosphere could itself lead to the further enhanced uptake of Hg(II) by plants (de Souza et al., 1999).

At sub-lethal levels, $HgCl_2$ reduces plant transpiration capacity in part by the binding of Hg(II) with sulfhydryl groups on root cell water channel proteins (mercury sensitive aquaporins), causing a physical obstruction to water flow (Maggio and Joly, 1995). An additional and possibly more significant effect of $HgCl_2$ on plant transpiration may be the direct toxicity of Hg(II) in slowing root cell metabolism, leading to a reduced aquaporin activity (Zhang and Tyerman, 1999). *merA* tobacco plants grown in hydroponic medium suffered a significantly less severe decrease in transpiration than the wild-type after the medium was spiked with Hg(II). The ability for the MerA protein to protect cells from general toxic effects of Hg(II) has been well documented. However, MerA may also clear mercury sensitive aquaporins which are physically blocked by sulfhydryl-bound Hg(II), converting the Hg(II) to Hg(0) and possibly drawing the Hg(0)

along with the bulk transpiration flow. Studies have suggested that the transpiration stream may provide the capacity to move Hg(0) (Kozuchowski and Johnson, 1978; Hanson et al., 1995). The ability of *merA* plants to convert root-absorbed Hg(II) to Hg(0) may enable these plants to more effectively move water (and concomitantly mercury) from Hg(II)-contaminated soil than the wild-type.

Suszcynsky and Shann (1995) have shown that uptake of airborne Hg(0) by tobacco results from absorption into leaves rather than adsorption onto leaf surfaces. In our study, wild-type and *merA* tobacco absorbed volatile Hg(0), confirming that *merA* plants retain an Hg(0) immobilization capacity typical of most plants. However, during a 5 h Hg(0) exposure, MerA activity was responsible for limiting the net absorbed mercury in *merA*-expressing plants to 1/8 the net absorption by the wild-type. The only known activity of the *merA* gene on mercury is the conversion of Hg(II) to Hg(0). Therefore, the difference between *merA* and wild-type plants in net mercury uptake is likely that amount of Hg(0) first immobilized to Hg(II), then reduced and re-volatilized by MerA. This suggests that Hg(0) immobilization in tobacco leaves occurs by the oxidation of Hg(0) to Hg(II). As expected, enzymes such as catalases (Du and Fang, 1983) that may oxidize mercury in wild-type plants, are present and functional in *merA* plants, however the competing reduction reaction is catalyzed by the MerA protein at a rapid rate causing *merA* tissues to have a lower net oxidative activity toward Hg(0) than the wild-type.

Both wild-type and *merA* plants exposed to volatilized Hg(0) for 5 h lowered their mercury concentrations after 24 h of growth in clean air. Surprisingly, this difference was most significant in the wild-type, indicating wild-type tissues do have a measurable endogenous Hg(II)-reducing activity. However, the +24 h wild-type still contained

almost 6 times the mercury concentration of the +24 h *merA* plants. The mercury remaining in tissues of +24 h *merA* plants may have been the small fraction of mercury that is immobilized to some form other than Hg(II), and which is consequently not available to MerA for reduction. For example, carbon-bound mercury would not be a substrate for *merA*.

Typically, mercury that is absorbed by roots remains relatively tightly bound there (Beauford et al., 1977; Leonard et al., 1998). The toxicity of highly-root-available Hg(II) to plants indicates that there is significant transport of mercury into root cells and not simply adsorption to the surface of root epidermal cells. *merA* plants have the capacity to convert the most typically-bound form, Hg(II) to the least reactive form, Hg(0). Mercury phytoremediation will be more effective if mercury is pumped through plants and released through leaves as Hg(0) than if Hg(0) is released from roots back into the soil. The tissue mercury concentrations of ungrafted and reciprocally grafted wild-type and *merA* roots and shoots grown in $HgCl_2$-spiked hydroponic medium indicate that mercury mobility through tobacco plants may be enhanced in tissues where the *merA* gene is expressed. Mercury concentrations were significantly lower in *merA* roots than wild-type roots regardless of aboveground genotype, demonstrating that mercury was removed from *merA* roots at a higher rate by the conversion of Hg(II) to Hg(0). The negligible quantity of mercury remaining in the hydroponic medium after the assay makes it unlikely that *merA* plants absorbed less Hg(II) than wild-type counterparts. On the contrary, results of the hydroponic transpiration assays indicate that when exposed to root-available Hg(II), *merA* plants should absorb more Hg(II) than the wild-type in parallel with a greater water uptake.

Tobacco expressing the *merA* gene in the root moved significantly more mercury to aboveground stems than tobacco with wild-type roots (Fig. 3.7B). A significantly higher stem and leaf mercury concentration in $wt_s/merA_r$ plants than in $merA_s/merA_r$ plants indicates that in the former, mercury was mobilized by *merA* roots, but was likely re-immobilized by wild-type aboveground tissues as Hg(II). As demonstrated by the differential net absorption of airborne Hg(0) by *merA* and wild-type tobacco plants, significant rates of both mercury oxidation and reduction occur in *merA* tissues, conferring on them a lower net mercury oxidative capacity than the wild-type. It is highly possible that mercury moves more efficiently through *merA* tissues than wild-type tissues by the repeated conversion between water soluble, reactive Hg(II) and the water insoluble, unreactive Hg(0) form. Mercury transport in wild-type tissues, though still present, may be slowed by a predominant mercury oxidative capacity causing mercury to remain in the Hg(II) form.

The capacity for wt/*merA* plants to move significantly more mercury to aboveground tissues than other genotype combinations shows that it may be possible to engineer a mercury sequestering plant using root-specific *merA* expression from a stable root specific promoter. Further engineering of aboveground tissues with enhanced expression of metal-binding proteins such as phytochelatins or metallothioneins could greatly enhance the ability of these tissues to bind and store large quantities of mercury. A mercury-sequestering plant could offer an additional option for site mercury remediation.

CONCLUSIONS

The simple constituitive Hg(II)-reductive capacity of *merA* plants may lead to a cumulative phenotype of mercury resistance, processing and growth responses that is very different from the wild-type, even at the physiological level. The mercury resistance that is conferred by the *merA* gene may help the plants maintain healthy physiological functions, while growing on mercury-contaminated substrates. Included in this is the ability for *merA* roots to grow into extremely high and localized concentrations of mercury instead of either avoiding such regions or limiting root growth. Once *merA* roots have penetrated soils of high mercury concentration, root-absorbed mercury is likely to be processed from roots more efficiently than in non-engineered plants. This may prevent damage or blockage to water and nutrient transport systems in roots, allowing *merA* plants to act as a continuously renewing mercury sink. Using efficient Hg(II)-reductive activity, *merA* plants may be capable of moving mercury from roots to leaves possibly through continued conversion and reconversion of the soluble but reactive Hg(II) to the insoluble but unreactive Hg(0). Once Hg(0) is released from the plant, *merA* plants are much less likely to re-absorb the volatile metal which would likely be carried away from the immediate area through the air. The simple addition of one gene, *merA*, to plants may provide adequate changes to mercury uptake and processing to make these plants capable of mercury phytoremediation under some circumstances. Analyses of *merA* plant growth and mercury processing on different mercury contaminated substrates will likely suggest other genetic modifications to create a highly efficient tool for mercury phytoremediation.

MATERIALS AND METHODS

Transformation

Nicotiana tabacum (tobacco) cv. Little Havana plants were previously transformed with the *merA9* gene using standard techniques for genetic engineering (Heaton et al., 1998). The *merA18* gene under control of the cauliflower mosaic virus (CaMV) *35S* promoter was introduced into the same variety of tobacco using transformation with the plant binary vector pVSTI (Malik and Wahab, 1993). The pVSTI vector also carries the kanamycin resistance gene, *nptII*. Tobacco tissues were transformed using the *Agrobacterium*-mediated sterile disk method (Horsch et al., 1988). Leaf sections of potentially transformed tobacco were incubated on plates with semisolid Murashige and Skoog (MS) medium (Gibco, Grand Island, NY) containing 1 mg/l BA, 0.1 mg/l NAA, 300 mg/l kanamycin and 400 mg/l timentin. Primary transformant shoots were transferred to semisolid MS plates with 100 mg/l kanamycin and 300 mg/l timentin and were grown until formation of healthy roots. All plates were incubated at 27˚C with a 16 h photoperiod. Plants were transplanted from plates to soil and grown to seed from which further generations were developed.

Transformation verification

PCR was used to confirm that putative transgenic plants contained either the *merA9* or *merA18* genes. Total genomic DNA was isolated from tobacco leaf tissues by the rapid alkali DNA screening method of Gilliland et al. (1998). Sense and antisense primers were designed to amplify a 222-bp long internal fragment of the *merA* gene

(Heaton et al., submitted). Tissue protein was extracted directly in 2X SDS sample buffer and samples were heated to 99˚C for 5 min. Proteins were resolved using SDS/12% PAGE (Laemmli, 1970) and electroblotted onto an Immobilon-P polyvinylidene fluoride membrane (Millipore, Bedford, MA). Monoclonal anti-MerA antibody mAb11F9 (Rugh et al., 1998) was used to react with membrane-bound protein after blocking with 5% dry milk/25% goat serum albumin (Sigma Co., St. Louis, MO) in TBSt buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20). Membranes were washed repeatedly in TBSt buffer to remove unreacted anti-MerA antibody and were then labeled with a secondary polyclonal sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Lifescience, Buckinghamshire, England). The blot was visualized using the enhanced chemiluminescence system (Amersham Lifescience, Buckinghamshire, England).

Germination and growth on toxic level Hg(II)-spiked semi-solid medium

In order to compare the survival and growth of *merA* and wild-type tobacco on normally toxic concentrations of Hg(II), approximately 10 *merA9* (line 2-1-A) or *merA18* (line 20-1-B) seeds and 10 wild-type seeds were placed in petri plates on semi-solid MS medium containing 60 μM $HgCl_2$. Seeds were positioned near one end of each plate and plates were incubated in a vertical position at 27˚C (16 h day) for 4 weeks (Fig. 3.2)

Root penetration of an HgS barrier

An experiment was designed to test the enhanced ability of plant roots expressing *merA* to penetrate a localized region of high Hg(II) concentration, simulating root

penetration of heterogeneous Hg(II) hot-spots in soil. Semi-solid MS medium was chosen for this experiment because it allows rapid, healthy growth of tobacco and allows roots to be viewed clearly. Initially, it was not known whether any mercury compound could be placed in large amounts in semisolid growth medium to create a barrier of Hg(II) that would not diffuse through the plate killing or severely inhibiting the growth of control (wild-type) plants. A preliminary experiment was carried out with two candidate compounds, HgO or HgS (cinnibar), both chosen for low dissociation constants. Approximately 50 mg of each was embedded in the center of separate petri plates containing 4-week-old *merA* and wild-type tobacco growing on MS semi-solid medium. Figure 3.3A shows an example of an MS plate embedded with HgO. Plants had been germinated on these plates in a line at one end of the plate, at a distance from that end of the plate equivalent to one quarter of the diameter of the plate (*merA* left, wild-type right). Plates were incubated in vertical position for 10 days. HgO dissociated readily and dissolved through the medium, killing all wild-type tobacco, but having little affect on the transgenics (Fig. 3.3A&B). Plants on plates embedded with HgS suffered no visible ill affect (not shown). It was thus determined that HgS was an appropriate mercury compound to create a localized (i.e. not diffusable) barrier of high Hg(II) concentration.

An equal number of wild-type and *merA* seeds were germinated on MS semi-solid growth medium in the same arrangement as the tobacco above. After plates were incubated vertically for 10 days, a 3 mm wide trough as deep as the agarose was cut through the agarose parallel to the line of germinated seedlings and one-third of the way from the opposite end of each plate. Each trough was lined with pure HgS (cinnibar)

powder and plates were replaced in the vertical position for 8 weeks, providing ample time for roots to grow the length of the plate. The number of roots of each tobacco genotype crossing the HgS line was counted at the end of the experiment. By this point, leaf growth had virtually covered the air space within the plate. For this reason, a more clearly viewed plate was generated by placing a smaller number (8) of each *merA18* or wild-type tobacco germinants at a shorter distance from the HgS trough (Fig. 3.3C). This provided a more rapid root encounter with the HgS, allowing a clearer visualization of those roots crossing the trough. After 2 weeks, this plate was photographed (Fig. 3.3D). On this unreplicated plate, 9 merA18 roots crossed the mercury trough compared to 2 of the wild-type. As no wild-type plants survived on the plates spiked with HgO, these plates are presented only as an illustration of *merA18* survival advantage over the wild-type on Hg(II)-infiltrated plates (Fig. 3.3A&B).

Effect of root-absorbed $HgCl_2$ on transpiration

In carrying out dissolved-Hg(II) hydroponics assays of the type presented in (Heaton et al., 1998), it was observed that wild-type plants transpired a lower volume of water than *merA* plants. In order to compare the differential effect of root-absorbed Hg(II) on wild-type versus *merA* tobacco transpiration, 6 *merA18* and 6 wildtype tobacco plants were placed in separate canning jars, each filled with 1 L of modified Clark hydroponics medium (Clark, 1982; Heaton et al., submitted). Each jar was aerated using a small aquarium pump and covered in aluminum foil to discourage algal growth. The experiment was conducted in a plant growth room maintained at 27˚C. Plants were exposed to a 16-h photoperiod under a light panel with alternating *Daylight Deluxe*

(Sylvania, USA) and *Plant and aquarium* (GE, USA) lamps. Light intensity was 125 μmol·m^{-2}·s^{-1} at the level of the lower leaves. Light composition was measured as: blue: 22.8 μmol·m^{-2}·s^{-1}; near IR: 12.5 μmol·m^{-2}·s^{-1}; red: 48 μmol·m^{-2}·s^{-1}. After 1 week of acclimatization, the hydroponic medium was replaced. Forty-eight hours later, the amount of water needed to refill each jar was measured by weight as an estimation of water loss via transpiration. This same quantified refilling was carried out every 48 h for the next 10 days. Immediately after the day 4 measurement, all hydroponic media were spiked with $HgCl_2$ to a final concentration of 25 μM Hg(II). Plants were grown in hydroponics for 6 days after the spiking.

Hg(0) absorption from air

Wild-type and *merA18* tobacco seeds were grown on semisolid MS medium, and transplanted 1 week after germination to independent 2.5 x 2.5 in pots containing Fafard 3B potting soil (Conrad Fafard, Inc., Agawam, Massachussetts). Plants were grown for approximately 3 weeks in a growth room (described above), after which wild-type and *merA* plants were transferred to a growth chamber with lower light intensity and temperature of 29˚C. Light intensity was 81.5 μmol·m^{-2}·s^{-1} at the level of the lower leaves. Light composition was measured as: blue: 15.6 μmol·m^{-2}·s^{-1}; near IR: 3.3 μmol·m^{-2}·s^{-1}; red: 21 μmol·m^{-2}·s^{-1}. Plants were placed in 2 blocks randomized by genotype (4 wild-type and 4 *merA* in each block) on a 2.5 cm thick section of styrofoam. A 40 ml glass vial containing enough Hg(0) to cover the bottom of the tube was placed without cap between the 2 blocks. At time zero, a transparent plastic dome (55 x 30 x 15 cm) with partially closeable vents was placed over the plants and the vial of mercury.

The dome was pressed into the styrofoam to minimize air flow from around the edge of the dome. Vents were left in "closed" position which allowed for some air exchange between the dome atmosphere and the outside atmosphere. Plants were allowed to incubate in this environment for 5 h. Temperature within the chamber was monitored by enclosing a visible thermometer for the duration of the incubation. Mercury vapor concentration in the chamber was monitored with 6 replicate samples of the headspace every hour using a Jerome 431-X mercury vapor analyzer (Arizona Instruments, Phoenix, Arizona). After the 5 h incubation was completed, the chamber lid was removed and the vial containing Hg(0) was re-capped. Half of each the *merA* and wild-type plants from each randomized block were removed from the growth chamber and aboveground tissues were harvested by severing the stem at the soil level. The half of the plants not initially harvested was allowed to remain on the growth chamber bench for an additional 24 h after which they were collected in the same fashion as the first harvest. Tissues were immersed in liquid nitrogen and ground to a fine powder using mortar and pestle. The resulting powder from each sample was completely lyophilized using a Labconco Freezone freeze-drying system (Labconco Corp., Kansas City, Missouri) and then acid digested with HNO_3:$HCLO_4$ (7:1, v/v) for 48 h. Acid extracts were analyzed for total mercury using Inductively Coupled Plasma Mass Spectrophotometry (ICP-MS).

Mercury transport to aboveground tissues: a grafting approach

This experiment was designed to assess the mobility of mercury through tissues of wild-type and *merA* tobacco. Wild-type and *merA* tobacco were germinated on semisolid MS growth medium. Two weeks after germination, plants were transplanted to Fafard

3B potting soil and grown to approximately 30 cm height. Shoots were cut from roots at a distance of 2.5 cm from the soil and all leaves were removed from shoots. Aboveground shoots were grafted back onto roots using the whip-and-tongue technique (Hartmann et al., 1990). Wild-type aboveground tissues were grafted to *merA9* roots (reciprocal graft), *merA9* aboveground tissues were grafted to wild-type roots (reciprocal graft), and same-genotype tissues were grafted together (same-genotype graft) to create all possible root/shoot genotype combinations. Parafilm (American National Can, Neenah, WI) was wrapped tightly around the graft junction. Small transparent plastic bags were placed over the newly grafted tops in order to reduce transpiration in this tissue and prevent shoot dessication. After 4 weeks of recovery and the formation of new leaves, tobacco plants were placed in hydroponic media using the materials and methods detailed for the transpiration assay above. Plants were allowed to acclimatize for 1 week and then the hydroponic media were spiked to 5 μM Hg(II). After 10 days, plants were harvested and roots (tissue below graft junction) were separated from shoots. Leaves were removed from shoots. Plant tissues were acid digested and analyzed for mercury content using materials and methods detailed above.

Statistical analyses

Two-tailed t-tests were used for statistical comparison between averages. An F-test ($p = 0.05$) was used on each pair of averaged values to determine whether a type 2 (equal variance) or type 3 (unequal variance) t-test was appropriate. All errors are expressed as as one standard error of the mean. All analyses were carried out using Microsoft excel (Microsoft Corp.)

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Figure 3.1. Confirmation of *merA* presence and MerA protein expression in transgenic *Nicotiana tabacum* (tobacco). A. PCR identification of tobacco plants expressing the *merA* gene. Nine lanes segregating from *merA18* line 20-1-A were assayed for the 210 bp band of the *merA* gene. The no-sample control lane (**-**) and the wild-type (wt) lane showed no reaction. **B. Western blot showing positive reaction of 3 plants from the *merA18* line 20-1-A.** The positive band appears at about 60 kD in *merA18* lanes and is not present in the 3 wild-type lanes. A coomassie gel confirms approximately equal loading of protein in wild-type versus *merA18* lanes.

Figure 3.1

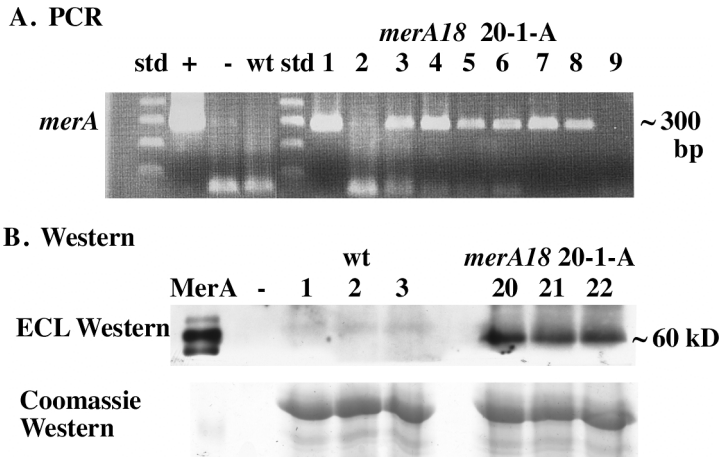

A. PCR
merA18 20-1-A
std + - wt std 1 2 3 4 5 6 7 8 9
merA
~300 bp
B. Western
wt
merA18 20-1-A
MerA - 1 2 3 20 21 22
ECL Western
~60 kD
Coomassie Western

Figure 3.2. *merA*-expressing *Nicotiana tabacum* (tobacco) are resistant to Hg(II). Wild-type (wt) and *merA* tobacco seeds were placed on semisolid semisolid 1x Murashige and Skoog (MS) medium containing 60 μM $HgCl_2$. Wild-type (left on each plate) did not germinate whereas *merA9* and *merA18* seeds germinated and grew well.

Figure 3.2

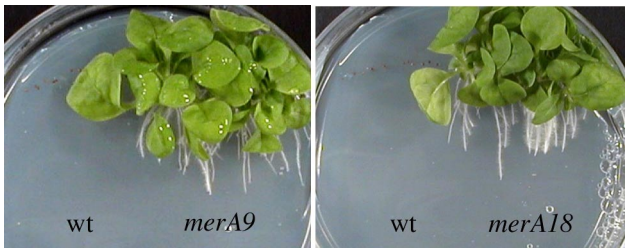


Figure 3.3. Root survival and penetration of agar embedded with HgO and HgS. A. Semi-solid 1x Murashige and Skoog (MS) medium with 4-week-old wild-type (left) and *merA Nicotiana tabacum* (tobacco) plants (right) was implanted with HgO (mercuric oxide)**. B.** After 10 days, wild-type were completely dead while *merA* plants appeared completely insensitive to the Hg(II). **C.** Eight wild-type (left) and eight *merA18* (right) germinants were placed on a semi-solid MS plate at one side of a groove lined with HgS. **D.** After 2 weeks the different abilities of wild-type and *merA* to cross a barrier of HgS was most clearly evident. This effect was statistically recorded in a longer-term experiment (Fig. 3.4).

Figure 3.3

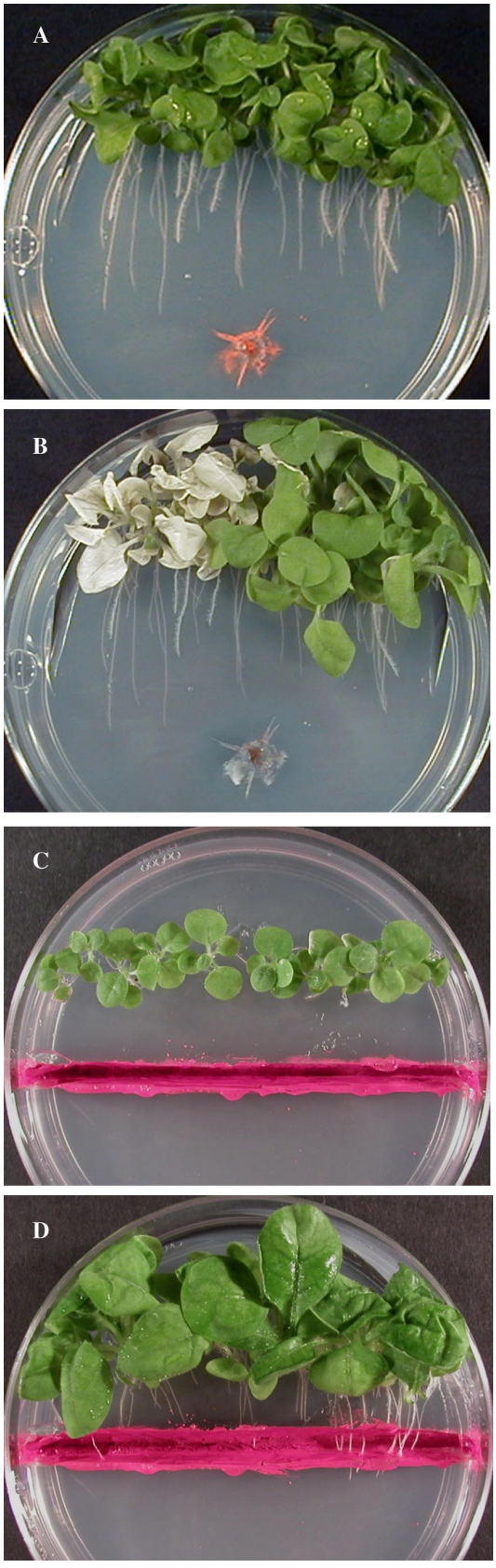


Figure 3.4. The number of *Nicotiana tabacum* (tobacco) roots growing across a HgS-lined groove. The mean number of roots per petri plate crossing the HgS groove are shown for a pair of each *merA9* and *merA18* plates, and then as means from all four *merA* plates (total *merA*). Counts are compared to those for an equal number of wild-type (wt) controls on each grouping of plates (*merA18*: n = 2, p = 0.072, *merA9*: n = 2, p = 0.095, *merA*tot: n = 4, p = 0.002). Errors are expressed as one standard error of the mean.

Figure 3.4

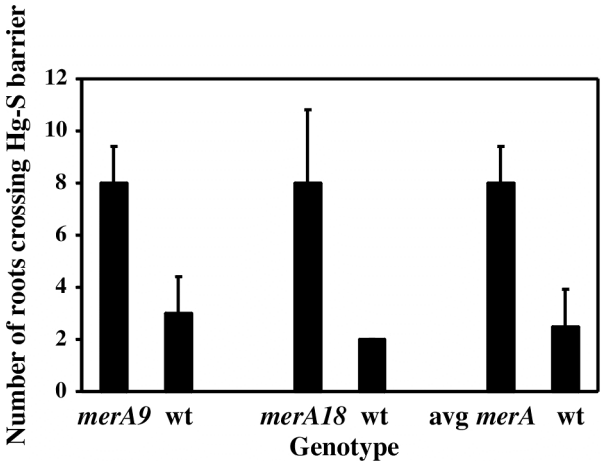

Number of roots crossing Hg-S barrier
12
10
8
6
4
2
0
merA9 wt
merA18 wt
avg merA wt
Genotype

Figure 3.5. The effect of Hg(II) on *merA* and wild-type tobacco transpiration. The mean water loss from hydroponic jars containing either *merA18* or wild-type tobacco plants was measured and expressed as a percentage of water loss during the 48 h period preceeding day 0 (± 1 standard error, n = 6). Immediately after the day 4 measurement, each hydroponics medium was spiked to a final concentration of 25 μM Hg(II) using $HgCl_2$.

Figure 3.5

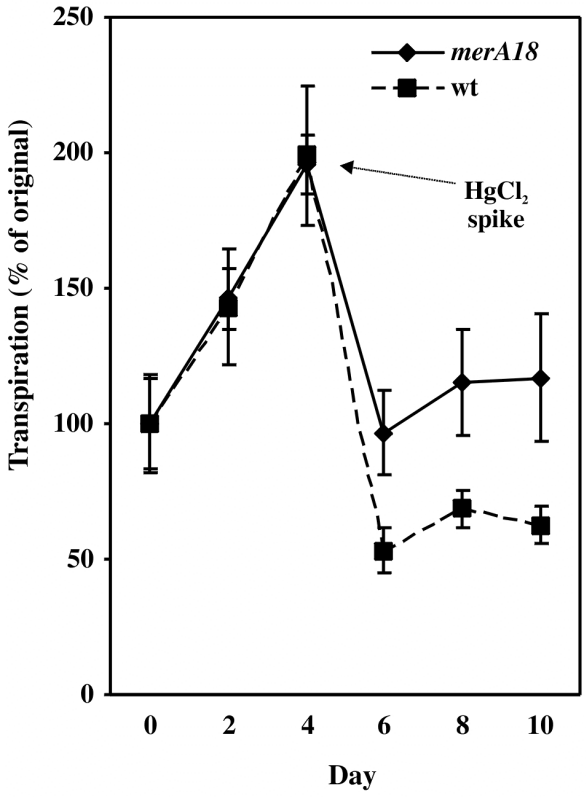

250
200
150
100
50
0
Transpiration (% of original)
0
2
4
6
8
10
Day
merA18
wt
HgCl2
spike

Figure 3.6. Net uptake and retention of Hg(0) vapor by wild-type and *merA* tobacco. The wild-type and *merA18* bars represent mercury concentrations in aboveground tissues of plants harvested after a 5 h Hg(0) exposure. Hg(0) concentration in the chamber rose from 0 to 54.3±1.4 $\mu g \cdot m^{-3}$ in the first hour and gradually increased for the duration of the assay to a final and maximum concentration of 74.8±6.8 $\mu g \cdot m^{-3}$. The wt (+24 h) and *merA18* (+24 h) bars represent mercury concentrations in tissues of plants grown for 24 h in clean air after the 5 h Hg(0) exposure (± 1 standard error, n = 4).

Figure 3.6

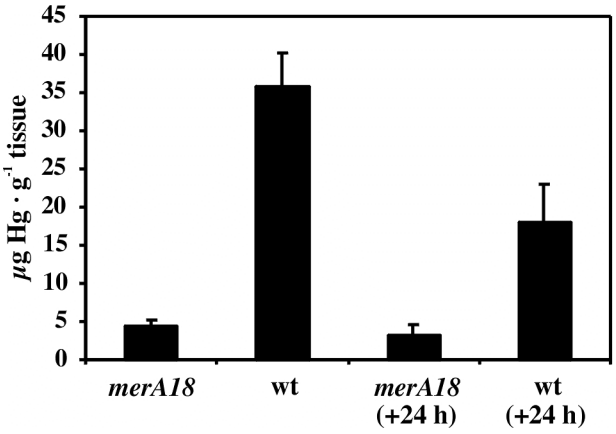
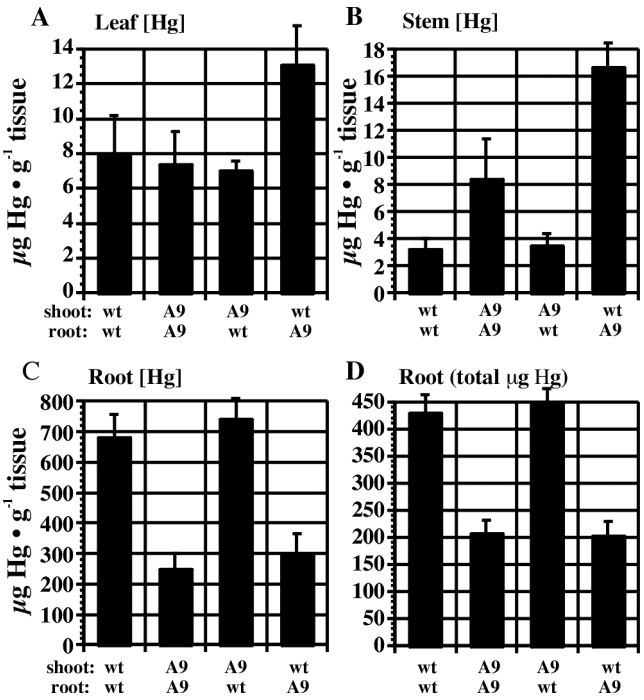

45
40
35
30
25
20
15
10
5
0
μg Hg · g-1 tissue
merA18
wt
merA18 (+24 h)
wt (+24 h)

Figure 3.7. Increased mercury accumulation in wild-type tobacco shoots grafted to *merA* tobacco roots. Values are mean Hg concentrations (± 1 standard error, n = 5) in leaves **(A)**, stems **(B)**, and roots **(C)**, and total Hg accumulation in roots **(D)** of *Nicotiana tabacum* (tobacco) plants. Values are shown for ungrafted plants or plants reciprocally grafted at the lower stem to have *merA9* (A9) shoot on wild-type (wt) root or wild-type shoot on *merA* root**.** Plants were grown for 10 days in $HgCl_2$ spiked growth medium**.**

Figure 3.7


A
Leaf [Hg]
B
Stem [Hg]
C
Root [Hg]
D
Root (total μg Hg)
μg Hg • g-1 tissue
shoot: wt A9 A9 wt
root: wt A9 wt A9

CHAPTER 4

IONIC MERCURY RESISTANCE OF *MERA* TRANSFORMED *POPULUS DELTOIDES* GROWN ON MERCURY SPIKED SOILS

Mercury pollution is most dangerous in waterlogged or frequently inundated soils and sediments where inorganic forms can be microbially converted to neurotoxic methylmercury. Phytoremediation may be an effective strategy to remove mercury from these habitats. *Populus deltoides* (Eastern cottonwood), which tends to live in riparian or other mesic soils has been engineered with the *merA* gene. The *merA* protein encoded by this gene catalyzes the detoxification of Hg(II) to Hg(0) which then volatilizes from the plant. Wild-type (non-engineered) and *merA* cottonwood were planted in Georgia field soil spiked to 420 ppm with Hg(II). Wild-type plants died within a few days whereas *merA* plants survived until a pre-planned harvest three months after planting. On soils with no mercury, wild-type grew significantly taller than the *merA*. To compare the differences in growth and mercury processing of wild-type and *merA* cottonwood in soils that were not acutely toxic to the wild-type, plants of each genotype were grown for 3 months in soil containing 0, 4, 8 and 40 ppm Hg(II). *merA* cottonwood were of significantly greater biomass than wild-type, as measured by final tissue dry weight, in the 40 ppm soil only. This indicates that Hg(II) resistant *merA* plants gain a growth advantage over the wild-type only as soil mercury concentrations increase. Mercury

concentrations in aboveground tissues of these plants indicate that the mercury resistance of *merA* cottonwood is likely a result of the reduction of Hg(II) to volatile Hg(0).

INTRODUCTION

Mercury pollution is an environmental problem primarily because the most common mercury chemical forms (inorganic mercury) can be converted to the highly neurotoxic methylmercury under suitable conditions. Sulfate-reducing bacteria that inhabit anaerobic, usually waterlogged soils are the primary facilitator of this process (Compeau and Bartha, 1985). Methylmercury can be biomagnified through the food chain to dangerously high levels in predatory animals, including humans (Huckabee et al., 1979). Phytoremediation, the use of plants to extract and then detoxify or sequester pollutants, may be a practical method to clean mercury or methylmercury pollution in water or soils (Meagher et al., 2000). Studies with *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco) demonstrated that transgenic model plants expressing a modified bacterial mercuric ion reductase gene (*merA9*) could detoxify mercury by converting the more toxic and reactive ionic form, Hg(II), to the less toxic elemental mercury, Hg(0) (Heaton et al., 1998; Rugh et al., 1996). The *merA9* gene and a further modified version of *merA*, *merA18,* were introduced into a tree species, *Liriodendron tulipifera* demonstrating that the same Hg(II) resistance and Hg(0) volatilization capacities could be conferred to a woody species (Rugh et al., 1998).

Eastern cottonwood (*Populus deltoides*) trees were engineered with the *merA9* or *merA18* genes to investigate the potential of a more flood-tolerant tree for mercury

phytoremediation in riparian or aquatic habitats. Eastern cottonwood is one of the fastest-growing trees in North America when growing in riparian habitats. On rich alluvial soils in the Mississippi valley, young trees may grow as much as 1.5 m in height and 2.5 cm in diameter each year for the first 25 – 30 years (Harlow et al., 1996). Eastern cottonwood is also amenable to propagation via rooted cuttings (Cooper, 1990), tissue culture manipulation (Coleman and Ernst, 1989) and genetic engineering (Dinus et al., 1995; Han et al., 2000). Other studies have shown that roots of eastern cottonwood can facilitate degradation of trichloroethylene (TCE) by microbially-mediated reductive dechlorination (Jones et al., 1999) and that significant TCE uptake and biotransformation occurs in hybrid cottonwood itself (Gordon et al., 1998). These abilities are important because many heavy metal polluted sites are also contaminated with organic pollutants such as TCE (Meagher et al., 2000).

Transgenic *merA9* and *merA18* shoots cultured in medium containing 25 μM $HgCl_2$ showed normal growth and rooted, while wild-type shoots were killed. When the transgenic cottonwood plantlets were immersed in Hg(II), they emitted 2-4 times the amount of Hg(0) relative to wild-type plantlets (Che et al., submitted). In order to test the *merA* activity of transgenic cottonwood in an manner which could more closely resemble field conditions *merA18* and wild-type controls were grown in field soils spiked to different concentrations of Hg(II). The growth, mercury resistance, and mercury processing of *merA* cottonwood on Hg(II)-spiked soils will be explored here.

MATERIALS AND METHODS

Lethal level mercury contamination

Plantlets of one *merA18* transclone (*merA18-7*), and the wild-type (C175) were produced by harvesting 1-2 cm long shoots from proliferating shoot cultures and rooting them in moistened Fafard #2 peat-based potting mix in Hillson-type Rootrainers (Spencer Lemaire, Canada). Plantlets were kindly supplied by Scott Merkle of the Daniel Warnell School of Forest Resources, University of Georgia, Athens, GA, USA. Cuttings were rooted in a humidifying chamber under cool white fluorescent lights (100 μmol·m^{-2}·s^{-1}). After hardening-off, plantlets, which ranged from 10-15 cm in height, were replanted in field soil (Cecil) from Watkinsville, GA, in the same Hillson-type Rootrainers. Ten plants of each genotype were planted in clean soil and in soil containing 415.94±21.35 ppm Hg(II) as $HgCl_2$. Soil was spiked in 1998 with $HgCl_2$ by the method of Heaton (1998) and refrigerated in the interim. Plants in soil were placed in a growth chamber under a light panel of one 75 W *Gro-Lux* (GE, USA) and thirteen 60 W *Cool White Supersaver* (Sylvania, USA) lamps. Light intensity was 125 μmol·m^{-2}·s^{-1} at the level of the lower leaves, with a 16 hour photoperiod. Light composition was measured as: blue: 22.8 μmol·m^{-2}·s^{-1}; near IR: 12.5 μmol·m^{-2}·s^{-1}; red: 48 μmol·m^{-2}·s^{-1}. Plants were bottom-watered daily to avoid leaching of Hg(II) from the soil. Heights of all surviving plants were measured after two weeks and at the end of each month for 3 months. Soil settling and plant transplantation stress prevented the accurate height measurement of plants in the first two weeks after transplantation.

Sub-lethal level mercury contamination.

Plantlets of the same genotypes as above were prepared and grown using the methods described above. In this experiment, 4 plants of each genotype were grown in soils originally spiked to 0, 50, 100 and 200 ppm Hg(II) in 1998 and refrigerated in the interim. Actual Hg(II) concentrations at the beginning of this experiment were 0, 4.24±0.21, 8.11±0.52, and 40.10±5.07 ppm, respectively. The losses of Hg(II) by soils between spiking and this experiment were likely the result of chemical or microbial reduction of Hg(II) and subsequent volatilization of the resulting Hg(0). Plants were grown in Hillson-type Rootrainers plugged at the bottom with cotton to prevent soil from washing out. Plants were top-watered to discourage roots from escaping from pots and were fertilized weekly with approximately 10 ml of 1/2x Peters 20-20-20 fertilizer with micronutrients, outdoor recipe, (United Industries Corp., St. Louis, MO). As with the previous soil experiment, heights of all surviving plants were measured after two weeks and at the end of each month for 3 months. However, at the end of this experiment, plants were harvested, aboveground tissues were lyophilized and weighed. Differences in height growth and biomass between *merA* plants and wild-type plants were analyzed by 2-tailed t-tests using Excel (Microsoft Corp.). Lyophilized tissue was ground in liquid nitrogen using mortar and pestle, was digested with 7:1 HNO_3:$HClO_4$ and was analyzed for mercury content using ICP-MS (Inductively Coupled Plasma- Mass Spectrophotometry). The tissue preparation and digestion was carried out using the procedure of Suszcynsky, 1995.

RESULTS

Two experiments were conducted to compare the abilities of *merA* and wild-type cottonwood plantlets to survive in mercuric ion-contaminated soil. In the first experiment, wild-type and *merA18-7* cottonwood were grown in a Cecil soil containing either no mercury or a lethal concentration (416±21 ppm) of Hg(II). Within 48 hours, wild-type plants were visibly withered compared to the plants with the *merA* construct. After one week, all but one of the wild-type plants were completely defoliated. By the end of the second week, all wild-type plants were dead, whereas all of the *merA* plants were still living and only slightly visibly affected by mercury (Fig. 4.1). Plant height measurements at 2 weeks, and at the end of the first, second and third months are shown in Figure 4.2. Plants of both genotypes on both soil types began the experiment at similar heights. After two months, wild-type plants had grown significantly taller than *merA* plants on soils with no mercury ($n = 4$, $p = 0.011$), indicating that *merA* trees might only compete with wild-type trees in soils where mercuric ion is present. Wild-type plants remained significantly taller than *merA* counterparts at the month 3 (final) height measurement ($n = 4$, $p = 0.018$). In mercuric ion-contaminated soil that killed wild-type plants, *merA* plants survived although they grew little over the three months.

Because results from the lethal level mercury experiment indicated that wild-type cottonwood plants could not survive Hg(II) concentrations approaching 420 ppm, while *merA*-expressing plants could, a second experiment was initiated in which plants of both genotypes were grown for three months in soils with lower mercury concentrations (0, 4±0.2, 8±0.5, and 40±5 ppm). In this experiment, no significant differences in height

growth among different lines grown on different soil concentrations of mercuric ion were found after 3 months of growth (data not shown). Similarly, final biomass of wild-type and *merA* plants was not statistically different on any of the soils, except for the most heavily mercury contaminated (40.10±5.07 ppm), where *merA18-6* plants were of higher final biomass than wild-type plants (Fig. 4.3; t-test, $p = 0.016$). While the wild-type plants did not accumulate significantly more biomass than the *merA* plants on soils with low or no mercury, Figure 4.3 indicates a possible trend in this direction. Overall, the growth/biomass results of the 2 soil experiments indicate a subtle growth disadvantage to the *merA* plants when mercury is not present in the environment or is present in low concentrations, but a statistically significant growth advantage as soil mercury concentration reaches higher levels.

Aboveground mercury tissue concentrations at the end of the assay were significantly lower in the *merA* than the wild-type plants (Fig. 4.4) in the 0 ppm (t-test, $p = 0.057$) and 4.24 ppm (t-test, $p = 0.014$) treatments only. A lower Hg(II) concentration in *merA* tissues is an indication that more mercury is being reduced to volatile Hg(0) by these plants than the wild-type.

DISCUSSION

The two assays with contaminated soil are the first to be reported for trees transformed with heavy metal resistance genes. While the assay on soil with a lethal mercury level presented a striking demonstration of the ability of *merA* to protect cottonwood trees from mercuric ion, the wild-type trees died so rapidly that they

provided no height growth or biomass accumulation data for comparison to the *merA* trees. Results from the assay on soils with sub-lethal mercury levels, while not as visually striking as the first assay, indicated the presence of a critical point somewhere between 8 and 40 ppm Hg(II) in this particular soil. At Hg(II) concentrations above this critical point, the processing of Hg(II) by MerA allows the transgenic trees to outperform wild-type trees.

A significantly lower concentration of mercury in the tissues of *merA* cottonwood compared to the wild-type on 0 and 4.24 ppm Hg(II) soil indicates that Hg(II) is being processed to Hg(0) and being volatilized by these plants at a greater rate than the wild-type. Earlier studies have provided no evidence to suggest that plants with the *merA* gene have reduced root uptake of mercury (Heaton et al., 1998; Rugh et al., 1996). The presence of mercury in plants grown on 0 ppm soil indicates that Hg(0) evolved from Hg(II)-spiked soil/plant systems was likely absorbed stomatally and oxidized to Hg(II) by all cottonwood in the 0 ppm soil and re-reduced to Hg(0) by the *merA* plants only. Recent studies by Heaton (Chapter 3) have confirmed this phenomenon with tobacco. The lack of statistical difference between genotypes in the final plant mercury concentrations in the 8.11 and 40.10 ppm soils may be a result of wild-type plants losing mercury-containing leaves to a greater extent than *merA* plants on the soils of higher Hg concentration. In the case of the 40.10 ppm treatment, this is supported by the fact that biomass in wild-type plants was significantly lower than that of the *merA* plants though the heights (data not shown) were not significantly different.

In conclusion, we have demonstrated that transgenic eastern cottonwood trees expressing a bacterial mercuric ion reductase enzyme were able to grow in the presence

of a Hg(II) concentration lethal to wild-type plants. Furthermore, there seems to be a critical point between 8 and 40 ppm Hg(II), for the soil used, above which *merA* plant mercury resistance led to greater biomass accumulation than in the wild-type. Below this concentration, wild-type cottonwood may have a slight growth advantage over the *merA* cottonwood. Finally, *merA* cottonwood may process significantly more mercury from growth substrates than the wild-type, though these experiments were only able to show a difference in the two soils of lowest mercury concentration. Cottonwood engineered with *merA* offers great promise for enhancing the efficiency of mercury volatilization from mercury-contaminated soils, semi-aquatic ecosystems and wastewater. Future investigations will determine whether these transgenic plants are capable of converting Hg(II) to Hg(0) efficiently in the field.

ACNOWLEDGEMENTS

The authors wish to thank Dr. Dayton Wilde and Mead-Westvaco Corp. for supplying plant materials and advice on cottonwood culture and transformation and Dr. William Randle for sharing his equipment. Thanks to Scott Merkle, Dongsheng Che, Amparo Lima for assistance with much of this work. Thanks also to Bruce Haines for helpful criticism in the preparation of this chapter. The research reported here was supported by a grant from the U.S. Department of Energy (DE-FC09-93R18262) to the South Carolina Universities Research and Education Foundation though a subcontract with the University of South Carolina (USC 98-414). Andrew Heaton was supported by

a STAR Fellowship from the United States Environmental Protection Agency (U-915647-01-0).

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Figure 4.1 After 2 weeks, *merA* Eastern cottonwood plantlets survive and the wild-type die on soils contaminated with 416 ppm mercuric ion. **A.** C175 (wild-type) plantlets and **B.** *merA18-7* plantlets.

Figure 4.1

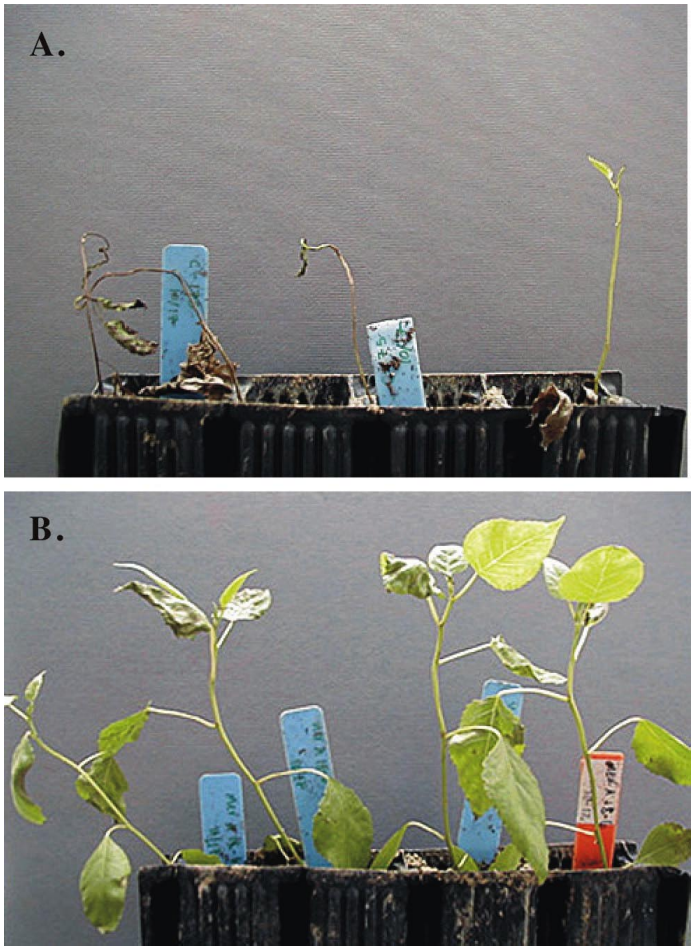


Figure 4.2 Height growth of transgenic *merA* and wild-type Eastern cottonwood plantlets on soil with or without mercuric ion contamination. Values shown are mean heights of 4 plantlets of each one wild-type (wt) clone and one *merA18* transgenic clone (merA18-7). Measurements were made after 2 weeks, 1 month, 2 months and 3 months in either uncontaminated soil (e.g. wt, merA18-7), or soil contaminated with 416 ppm Hg(II) (e.g. Hg wt, Hg merA18-7).

Figure 4.2

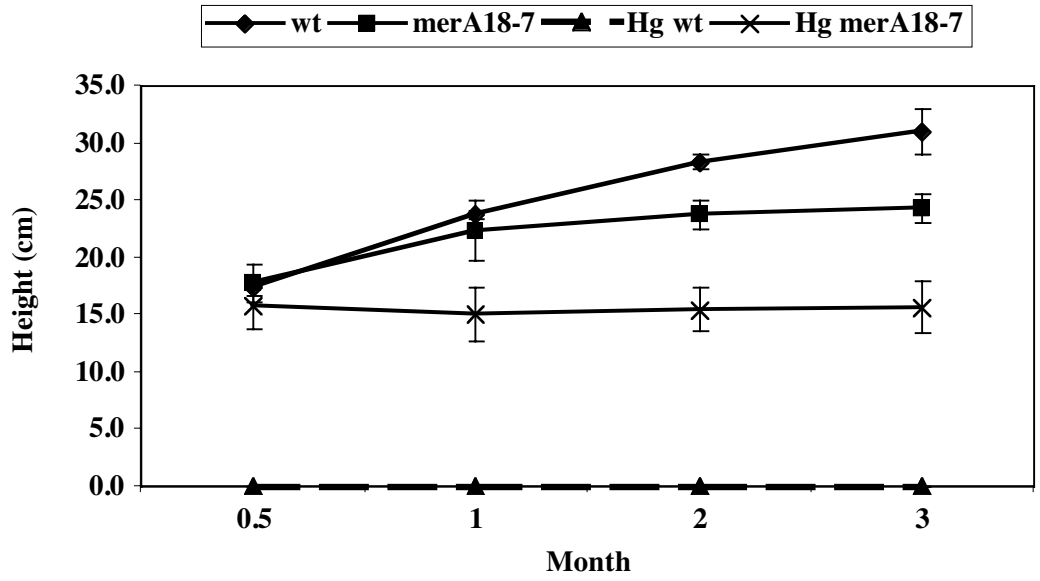

wt
merA18-7
Hg wt
Hg merA18-7
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Height (cm)
0.5
1
2
3
Month

Figure 4.3 Above-ground biomass of *merA* and wild-type Eastern cottonwood plantlets following 3 months of growth in soil with four levels of mercuric ion contamination. Values shown are mean lyophilized weights of above-ground tissues of 4 plantlets of each the wild-type (wt) clone and one *merA18* transgenic clone (*merA18*-6) grown on soils having four different concentrations of Hg(II). Error bars indicate one standard error of the mean.

Figure 4.3

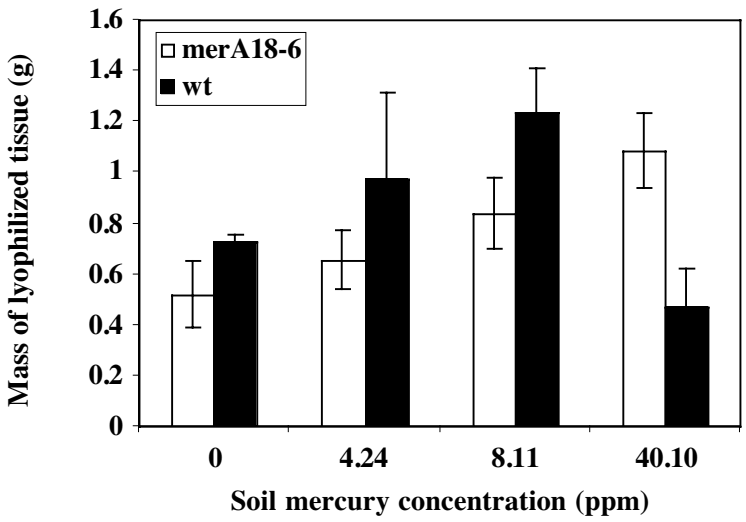
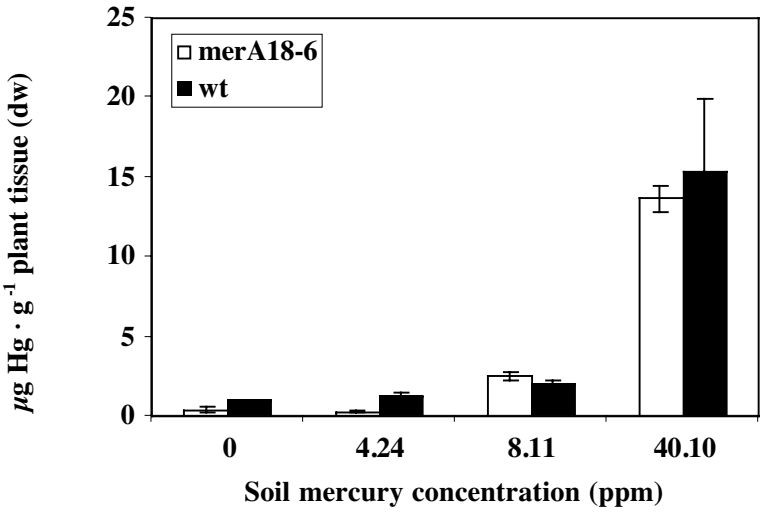

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
merA18-6
wt
Mass of lyophilized tissue (g)
0
4.24
8.11
40.10
Soil mercury concentration (ppm)

Figure 4.4 Mercury concentrations in aboveground tissues of transgenic *merA* and wild-type eastern cottonwood plantlets following 3 months of growth in soil with four concentrations of mercuric ion contamination. Error bars represent one standard error of the mean.

Figure 4.4


25
20
15
10
5
0
merA18-6
wt
μg Hg · g-1 plant tissue (dw)
0
4.24
8.11
40.10
Soil mercury concentration (ppm)

CHAPTER 5

TOWARD DETOXIFYING MERCURY-POLLUTED AQUATIC SEDIMENTS USING RICE GENETICALLY ENGINEERED FOR MERCURY RESISTANCE[1]

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[1]Heaton, A.C.P., C.L. Rugh, N.J. Wang, R. Kim, and R.B. Meagher. Submitted to Environmental Toxicology and Chemistry, 9/16/02.

ABSTRACT

Plant species expressing the bacterial mercuric reductase gene, *merA*, convert ionic mercury, Hg(II), from growth substrates to the less toxic metallic mercury, Hg(0). This activity confers plant mercury resistance and removes mercury from the plant and substrates through volatilization. Our goal is to develop plants to intercept and remove Hg(II) from polluted aquatic systems before it can undergo bacterially-mediated methylation to the neurotoxic methylmercury (MeHg). Therefore, *Oryza sativa* (rice) has been genetically engineered to remediate mercury-contaminated aquatic sediments. The *merA* gene under the control of a monocot promoter was introduced into *Oryza sativa* by particle gun bombardment. This is the first monocot and first wetland-adapted species to express the gene. The *merA*-expressing rice germinated and grew on semisolid growth medium spiked with sufficient Hg(II) to kill the non-engineered (wild-type) controls. To confirm that the resistance mechanism was the conversion of Hg(II) to Hg(0), germinants of *merA*-expressing *O. sativa* were grown in Hg(II)-spiked liquid medium or water-saturated soil media and were shown to volatilize significantly more Hg(0) than wild-type counterparts. Further genetic manipulation could yield plants with increased efficiency to extract soil Hg(II) and volatilize it as Hg(0) or with the novel ability to directly convert methylmercury to Hg(0).

Keywords--wild rice, *Oryza sativa*, mercury, phytoremediation, genetic engineering

INTRODUCTION

We present a genetically engineered monocot, rice, for the purpose of processing mercury from contaminated sediments. Mercury pollution has led to large numbers of human fatalities and illness and has had a significant impact upon the environment throughout the world [1]. Mercury contamination may occur from direct discharges, as was the case of the 1950s disaster in Minamata, Japan [2] and which is ongoing with gold mining practices in the Amazon River basin [3], or by particulate deposition of ionic mercury (Hg(II)) to remote locations [4]. Hg(II) in watersheds and aquatic areas may undergo conversion to methylmercury (MeHg), the most toxic naturally-occurring mercury form. MeHg formation is influenced by a variety of biotic and abiotic factors, though is mediated primarily by sulfate-reducing bacteria in anoxic sediments [5]. MeHg may then accumulate to greatly elevated and harmful concentrations at higher trophic levels via biomagnification [6]. The well-documented ecotoxicology of mercury pollution has led to reductions in industrial and agricultural use and intensive remediation efforts. However, the majority of large, mercury-polluted sites remain contaminated and remain an environmental threat.

The standard approaches for treating mercury-polluted sites, typically excavation and landfilling, are very costly because mercury pollution may occur over large areas of wetlands or along banks of extensive river systems. Site excavation can also result in the mobilization of soil-bound mercury into adjacent bodies of water. For these reasons, biological-based methods of mercury remediation have been investigated as affordable and practical alternatives. Discovery of resistant bacteria at mercury-polluted habitats

suggested the possibility of bioremediation for the cleanup of such sites [7]. Unfortunately, this strategy is usually ineffective for mercury removal in natural environments, because bacterial-soil enrichments are difficult to sustain in situ due to their restrictive niches and instability under fluctuating conditions [8, 9]. Plants, on the other hand, have been shown to have measureable capabilities for extracting mercury from soils and even volatilizing it at low levels [10, 11]. Phytoremediation, the use of plants for pollution removal and detoxification, has proven effective against a number of soil and aqueous pollutants [12, 13]. However, plants grown with roots exposed to bioavailable, easily root-absorbed mercury typically only acquire miniscule amounts of mercurial compounds in aboveground tissues [14]. Furthermore, plants are not known to naturally possess the ability for efficient detoxification of hazardous ionic or organo mercurials by conversion to Hg(0) that is shown by mercury-resistant bacteria. To overcome these limitations, we initiated a biotechnological strategy to develop plants for mercury phytoremediation.

The development of model transgenic plants for mercury detoxification has been described in a number of recent papers [15-19]. In each of these reports, one or both enzymes, mercuric reductase (MerA) and/or organomercurial lyase (MerB), of the bacterial mercury resistance (*mer*) operon [20] were successfully expressed in engineered plants to confer resistance to highly toxic mercurials in growth media. Resistance was conferred through the ability of *mer* plants to detoxify these compounds to the relatively inert, volatilized Hg(0). In particular, plants expressing the *merA* gene are able to convert Hg(II) to Hg(0) using electrons carried via cytoplasmic NADPH originally generated from photosystem I:

$$\text{Hg(II)} + \text{NADPH} \xrightarrow{\text{MerA}} \text{Hg(0)} + \text{NADP}^+ + \text{H}^+$$

Studies of *mer*-transformed plants utilized experimental model dicot species, such as *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum*), and yellow poplar (*Liriodendron tulipifera*), to examine the ability of plants to express bacterial *mer* genes. Because none of these species prefer the water-saturated soils most adversely affected by mercury pollution, we have transferred the bacterial *merA* gene into flood-tolerant, domesticated rice (*Oryza sativa*). *merA*-rice may facilitate phytoextraction of the bioavailable fraction of the Hg(II) reservoir from polluted sediments with subsequent conversion to relatively harmless Hg(0) vapor, thereby impeding MeHg biosynthesis and preventing its accumulation in the trophic cycle. A successful demonstration of the effectiveness of *merA*-rice would provide incentive for the incorporation of additional wetland species into this genetic engineering strategy and for the development of mercury remediative plant communities in the most environmentally sensitive habitats.

MATERIALS AND METHODS

Plant material

Oryza sativa L. Japonica cv**.** TNG67 was provided by Dr. Li-Fei Liu (National Taiwan University, Department of Agronomy, Taipei, Taiwan). Rice embryos were cultured and transformed by a modification of a technique provided by R. Wu, Cornell University [21]. Mature seeds were de-hulled and surface sterilized with 70% ethanol for 2 min, then 30% Clorox (Clorox Corp., Oakland California) / 0.02% Tween 20 (Sigma,

St. Louis, Missouri) for 30 min, followed by 4 washes with sterile distilled water. The seeds were placed on LS medium [22], pH 5.8, containing 0.8% agarose, 30 g/l sucrose, and 2.5 mg/l 2,4-D. After 2 weeks, embryogenic calli that developed from the scutella were excised and transferred onto fresh medium and subcultured every 3 weeks. Eight weeks after rice culture initiation, embryogenic calli were transferred into AA_2 liquid medium [21] containing 3% sucrose, 2.0 mg/l 2,4-D, 0.2 mg/l kinetin, and 0.1 mg/l gibberellic acid (GA_3). The suspension cultures were maintained in the dark at 26°C on a rotary shaker at 120 rpm and the medium was replaced weekly. Rapidly dividing, friable calli were subcultured into a fresh flask by transferring 2 ml of fine cell suspension (<140 μm) into 35 ml of AA_2 liquid medium. Suspension cultures were subcultured weekly prior to bombardment.

Plasmids

Plasmid pTRA140 containing the *hph* gene [23], generously supplied by N. Murai (Louisiana State University), was co-bombarded with plasmid pAL77 [24] containing the *merA9* gene [18] under control of the maize ubiquitin promoter [25].

Particle bombardment

A two- to four-month-old suspension culture that had been subcultured 5 to 6 days in advance served as target cells for particle bombardment. Cell clumps less than 520 μM in size were placed on a 5.5 cm diameter Whatman #1 filter paper and washed twice with 1/2X AA_2 medium by vacuum filtration (modified from Cao et al.) [21]. For osmotic pre-conditioning, the filter-bound tissues were placed on dishes of semisolid

Chu-1 medium (recipe provided by Dr. Qiren Chu, Louisiana State University Rice Research Station) supplemented with 0.25 M mannitol [26]. These dishes were used for the bombardment experiments the following day. The Biolistic PDS-1000/He system (Bio-Rad; Hercules, CA) was employed for cell transformation using manufacturer's instructions. In brief, approximately 0.5 mg of gold microcarrier (1.0 μm) was coated with 2 μg of either *merA9* or control plasmid DNA for each single bombardment. The plated rice tissues were positioned 10 cm below the macrocarrier stopping plate and 1100 psi rupture discs were chosen to control the helium pressure. Each plate was bombarded twice.

Selection of transformants

One day after the bombardment, the cells were transferred to mannitol-free Chu-1 medium and maintained in dark at 26°C. After 7 days, the cells were transferred to the mannitol-free Chu-1 containing 30 mg/l hygromycin B for preliminary selection for 1 week (modified from Qu et al.) [27]. Hygromycin-resistant (hyg^r) colonies were purified by weekly transfer to fresh mannitol-free Chu-1 containing 50 mg/l hygromycin B. Established hyg^r calli were transferred to Murashige & Skoog (MS) regeneration medium (Gibco, Grand Island, NY) containing 3 mg/l BA, 0.5 mg/l NAA and 30 mg/l hygromycin B and maintained at 26°C under a photoperiod of 16 hrs at 80 μE. As plantlets were regenerated, they were transferred to rooting medium (1/2X MS containing 30 mg/l hygromycin B). When well-rooted plantlets were over 12 cm high, they were transferred to pots and grown to maturity in the greenhouse. Original independently tranformed plantlets were each given a unique line number.

Polymerase Chain Reaction (PCR) assay

To confirm putative transgenic plants by PCR, total genomic DNA was isolated from rice leaf tissues by the rapid alkali DNA screening method of Gilliland et al. [28]. Sense (5'- AGTGACCATTCTTGCACGCTCCACTCTCTT -3') and antisense (5'-TCGCATCCAGTGCCAGCTTGCGTGTGTT -3') primers were designed to amplify a 222-bp long internal fragment of the *merA* gene.

Protein western blot screening

Crude protein was prepared from *merA9*-transformed and non-engineered (wild-type) plants in a plant extraction buffer containing 5 mM EDTA, 10 mM $MgCl_2$, 10 mM NaCl, 25 mM Tris-HCl, pH 8.7, protease inhibitor tablet (Roche diagnostics, Mannheim). For western blot, extracts were denatured by adding an equal amount of 2X SDS sample buffer and boiling for 5 min and then were separated with SDS/10% PAGE [29]. Resolved protein was electroblotted onto an Immobilon-P polyvinylidene fluoride membrane (Millipore, Bedford, MA). Monoclonal anti-MerA antibody mAb11F9 [19] was used to react with membrane-bound plant protein for 2 hrs after blocking for 2 hrs with 5% dry milk/25% goat serum albumin (Sigma Co., St. Louis, MO) in TBSt buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20). Membranes were washed in TBSt buffer to remove excess anti-MerA antibody and were then labeled with a secondary polyclonal sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Lifescience, Buckinghamshire, England). The blot was visualized using the enhanced chemiluminescence system (Amersham Lifescience, Buckinghamshire, England).

Mercury resistance assays

Wild-type rice seeds (line TNG67) were placed on semisolid 7% agar MS growth medium (Gibco, Grand Island, NY) in petri plates containing different concentrations of Hg(II) in order to determine their sensitivity to Hg(II). It was determined that 150 μM Hg(II) was phytotoxic to wild-type. The *merA*-expressing rice assayed in the same way survived on concentrations of Hg(II) up to and exceeding 300 μM. Therefore, 250μM Hg(II) semisolid agar MS plates were used for resistance assays and to screen potentially *merA*-expressing seeds.

Mercury vapor assays

For determination of the Hg(II)-reducing ability of *merA*-rice, 1-week-old germinants of transformed (lines 2, 3, 4, and 15) or a wild-type line were incubated in 2 ml of assay medium [18] containing 250 μM $HgCl_2$. Incubation was carried out in a specialized reaction tube (Fig. 5.3A). The headspace from the reaction tube was evacuated into a Jerome 431-X mercury vapor analyzer (Arizona Instruments, Phoenix, Arizona) immediately after the seedling was placed in the medium (time zero) and was resampled each min for 10 min. The mercury evolution rates from the medium into the headspace were normalized by dividing the total number of nanograms of Hg(0) measured by the number of milligrams of seedling tissue in each assay.

Mercury processing by rice in hydroponics

In order to determine the Hg(II)-reducing capability of mature *merA* rice plants, tillers of pre-flowering, mature *merA*-expressing line 15 and wild-type rice (1.5 months

after germination) were separated and rooted in water. After 1.5 weeks of rooting, 8 *merA* and 4 wild-type rice plants (from tillers) were placed in separate, 1 L Ball canning jars (Alltrista Corp., Muncie, Indiana) which were then each filled with 1 L of Clark hydroponics medium [30] modified with 1x $FeSO_4$ chelate solution (Sigma chemicals, St Louis, MO) replacing the typical $Fe(NO_3)_3$ requirement. Each was aerated with an aquarium pump. Plants were placed in a growth chamber under a light panel with alternating *Daylight Deluxe* (Sylvania, USA) and *Plant and aquarium* (GE, USA) lamps. Light intensity was 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the level of the lower leaves, with a 16 hour photoperiod. Light composition was measured as: blue: 22.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$; near IR: 12.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$; red: 48 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plants were allowed to acclimatize to the hydroponics environment for 1 week, after which the hydroponics medium was replaced and spiked with $HgCl_2$ to a final concentration of 2 ppm or 10 μM Hg(II). After 10 days of treatment, plants were harvested, shoot and root tissues were separated, and each sample was ground in liquid nitrogen to a powder by mortar and pestle. Samples were completely lyophilized using a Labconco Freezone freeze-drying system (Labconco Corp., Kansas City, Missouri). Dried tissue samples were digested in 5 ml nitric acid:perchloric acid (7:1 v:v) per 0.5 g plant tissue for 24 hrs, diluted to 100 ml with dH_2O, and the acid extract supernatant was measured for Hg(II) using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Assays of mercury processing by rice in sediments

In order to compare the mercury-processing capabilities of *merA* and wild-type rice plants grown in mercury-containing sediments, tillers of mature *merA*-expressing

(line 7c) and wild-type rice were separated and rooted in water. After 1.5 weeks of rooting, 4 *merA* and 4 wild-type plants were planted separately in 0.5 L Ball canning jars (Alltrista Corp., Muncie, Indiana) containing approximately 400 ml of either mercury-contaminated soil from Oak Ridge, TN or a laboratory spiked middle-Georgia soil, each seived to 1 cm^2. In 1997, Oak Ridge soil was collected from a stream bank, homogenized, and analyzed for mercury concentration, which was determined to be 1064.46 ± 60.99 ppm. It was refrigerated until the beginning of this experiment, at which time the concentration was determined to be 945.31 ± 28.32 ppm. Laboratory spiked soil was spiked to 50 ppm in 1998 [17] and refrigerated until this experiment. Soils contained 35.18±2.74 ppm Hg(II) by the beginning of this assay. The mercury lost by either soil between the initial analyses or original spiking and the rice sediment experiments was likely lost to volatilization during the refrigeration. Jars were filled with enough water to cover the soil and to about an inch depth of excess water (approx 100 ml). Jars were refilled daily to the same level and fertilized weekly with 5 ml of 1x Peters 20-20-20 fertilizer with micronutrients (United Industries Corp., St. Louis, MO). Plants were grown in the same growth chamber with the same light environment as those in the hydroponics assay (above). After 3 months, jar contents were allowed to dry and plants were cut at the soil level and harvested. Plants were digested and analyzed for Hg(II) using the nitric/perchloric acid digestion and ICP-MS analysis detailed above. Root material was separated from the soil, rinsed in water and analyzed for mercury content using the same procedure as the aboveground tissue. Soil from each sample was dried to bench dryness and each sample was homogenized independently. Five grams of each soil

sample were digested with 8 ml of aqua regia for 3 days and digests were analyzed for Hg(II) using ICP-MS.

RESULTS

merA-rice production

Previous successes in expressing *merA* in dicot species all used the the Cauliflower Mosaic Virus (CaMV) *35S* promoter [16, 18, 19], which is often poorly expressed in monocots. Thirty-five independent rice plants transformed and regenerated with this *35S/merA9* construct failed to express MerA protein at more than trace levels (data not shown). Therefore rice calli were co-transformed with pAL77 carrying the *merA9* gene under control of the maize ubiquitin promoter (*ubi1p/merA)* and plasmid pTRA140, which encodes hygromycin resistance. Approximately 200 independent calli of putative transformants were recovered using hygromycin selection (Fig. 5.1A). Of these, 100 hygromycin-resistant lines regenerated to shoots (Fig. 5.1B). More than 50 of these healthy, green lines formed roots on hygromycin medium and were grown to maturity in soil (Fig. 5.1C).

Polymerase Chain Reaction (PCR) amplification of a region of the *merA* gene was used to confirm the presence of the un-selected pAL77 plasmid included in the co-bombardment. Small leaf sections from one or more tillers from each plant were assayed by PCR using *merA* gene primers. The majority of lines assayed for *merA* DNA were positive, as shown by the sample set of PCR reactions in Figure 5.2A. Of the Hyg^r plant lines examined, about 70% also contained the *merA* gene from pAL77, suggesting a

bombardment co-transformation frequency for these two plasmids of approximately 70%. When multiple tillers from the same plant were assayed (data not shown), all the tillers were usually of same genotype. In cases where only a subset of tillers assayed were positive for *merA,* it was presumed that the plant was a genetic mosaic containing both transformed and non-transformed cells. In these instances the *merA*-positive portions of the plant were excised and replanted in soil to eliminate germplasm lacking the *merA* gene.

Plants confirmed to contain the *merA* gene were assayed for expression of the MerA protein with a MerA-specific monoclonal antibody [31]. A typical western blot (Fig. 5.2B) shows five lines positive for the 56 KDa MerA protein. Two or more bands of about 56 kDa and smaller sizes are often seen on western blots of the MerA protein, because the 565 amino acid protein has a protease sensitive site [32]. About 75% of the rice lines containing the pAL77-encoded *ubi1p/merA* gene expressed the MerA protein in sharp contrast to the entirely negative results with the *35S* promoter construct.

Approximately half of the transformed plants (12-15) were fertile and capable of viable seed production (Fig. 5.1D). *merA*-expressing rice seeds were selected from wild-type segregants on semisolid agar plates containing 250 μM Hg(II). The wild-type seeds died on this concentration of Hg(II) within 5 days of germination (Fig. 5.1E, center). *merA*-expressing rice can grow indefinitely on this concentration of Hg(II) in semisolid growth medium (Fig. 5.1E, right). Sterile plants were propagated vegetatively and cuttings continued to show Hg(II) resistance. Successive generations from all positive *merA* lines displayed genotypic and reproductive stability and showed significant Hg(II) resistance compared to the wild-type. Wild-type rice seeds and plants were found to have

relatively high mercury resistance compared to other wild-type plant species (tobacco, *Arabidopsis*, yellow poplar, cottonwood), germinating and growing indefinitely on semisolid growth medium of up to 150 μM Hg(II), whereas most plant species we have examined previously die on concentrations of 25-65 μM Hg(II). On potting soils that contained no mercury *merA* Rice plants grew slightly less vigorously than the wildtype (Fig. 5.1C).

Mercury vapor assays

In order to demonstrate directly the mercury-processing activity of *merA*-transformed rice lines with positive MerA protein expression, small rice geminants were incubated separately in closed reaction bubblers, immersed in Hg(II) for 10 minutes. The results for *merA* lines 2, 3, 4, 15 and three wild-type plants are shown in Figure 5.3B. Hg(0) produced by conversion of Hg(II) entered the bubbler apparatus headspace and was sampled using the mercury vapor analyzer at 1 minute intervals for 10 minutes (Fig. 5.3A). Germinants of all *merA* lines showed substantially more Hg(0) volatilization than the wild-type germinants (Fig. 5.3B) indicating enhanced MerA, Hg(II)-reducing activity in these plants. There was substantial but reproducible variation in levels of Hg(0) volatilization among *merA* lines as observed in other studies [18].

Mercury processing by mature *merA* rice

Wild-type and line 15 *merA* rice were grown in Hg(II)-spiked hydroponics medium for a further comparison of Hg(II)-reducing capabilities. The hydroponics system enabled the addition of a quantifiable, highly root-available spike of Hg(II) to the

liquid growth medium. A negligible fraction of the original 2 ppm Hg(II) concentration (less than 1% of original concentration) remained in the hydroponics medium for all plants of both genotypes by the end of the 10-day assay. This was determined by treating the final medium with $SnCl_2$ to reduce Hg(II) to Hg(0) and then analyzing the resulting mercury vapor with a mercury vapor analyzer (data not shown). The majority of the Hg(II) removed from the hydroponics medium by wild-type plants was found to remain bound to the roots. The *merA* plants had approximately half the mercury in root tissues and aboveground tissues than the wild-type by the end of the experiment (Fig. 5.4A), suggesting that the *merA* plants had reduced more Hg(II) to Hg(0) than the wild-type. Both wild-type and *merA* plants contained much lower concentrations of Hg(II) in aboveground tissues than in roots (Figs. 5.4A and 5.4C).

Wild-type rice and rice of a moderately-expressing *merA* line, 20, were grown for 3 months on 2 different Hg(II)-containing "flooded" soils. This experiment was designed to compare Hg(II)-processing capabilities on growth substrates with less available Hg(II) than in hydroponics. Plants grown on these soils translocated less mercury into roots and aboveground tissues than plants in hydroponics medium (Figs. 5.4B and 5.4C). The soil from a contaminated site in Oak Ridge, TN contained much more Hg(II) than the laboratory spiked soil (Table 1), possibly leading to a much higher Hg(II) concentration in and/or bound tightly to the roots of plants grown on the Oak Ridge soil than the lab-spiked soil. However, shoot mercury concentrations of rice plants grown on the two soils were only slightly higher per genotype in the Oak Ridge soil, possibly indicating that bioavailable Hg(II) may not have been as different in the two soils as was the total soil Hg(II) concentration. The *merA* root and shoot tissues contained significantly lower final

mercury concentrations than the wild-type in the Oak Ridge soil. Because the activity of *merA* results in the volatilization of Hg(0), it can be inferred that the lower mercury levels in *merA* plants resulted from the loss of Hg(0) from these plants. There was no statistically significant difference in the final soil concentrations relating to which genotype was grown in the soil over the course of the three-month experiment (data not shown).

DISCUSSION

Our strategy has been to explore the *merA*-rice-mediated detoxification of the chemical precursor to methylmercury, Hg(II). The bacterial gene *merA* mediates the chemical reduction of Hg(II) to Hg(0) in genetically engineered rice plants. *merA*-expressing plants such as rice, which can grow large root mats and are capable of living partially immersed in aquatic or marine sediment should have a greater capability to block methylmercury production than would be possible with *merA*-expressing plant species more suited to upland, terrestrial habitats. With further genetic manipulation, rice expressing both *merA* and *merB* could facilitate complete enzymatic detoxification of methylmercury as demonstrated in *Arabidopsis* [15, 16].

Approximately half of the transformed, *merA*-expressing rice plants were found to be sterile. This blocks sexual propagation of sterile *merA* lines, however rice can be easily propagated by vegetative cuttings. Sterility has positive implications for the use of *merA* rice in field applications. If sterile lines were propagated through clonal cuttings and placed at a mercury-contaminated site, there would be no risk of pollen or seed

distribution and escape. The only containment concern would involve lateral growth of the originally planted rice, which could be easily controlled through appropriate field management practices. Furthermore, *merA* rice seem to grow slightly less vigorously than the wildtype in soils that contain no mercury (Fig. 5.1C). As a result the *merA* plants may not successfully compete with native plants outside of a Hg(II)-impacted habitat, or may become less competetive than natives once a habitat has been cleaned of Hg(II).

merA rice plants were shown to be highly resistant to Hg(II) in laboratory conditions relative to wild-type counterparts. This ability should confer a selective advantage to *merA* over wild-type rice in soils or sediments that contain extremely high concentrations of mercury. Furthermore, because mercury contamination in soils is found to be highly heterogeneous, the roots of *merA* rice may grow into and through highly mercury-contaminated hotspots. We have observed that *merA*-expressing tobacco plants have a greater ability than the wild-type to grow through localized regions of extremely high mercury concentrations in a semisolid matrix (Heaton, in prep.). In a similar way, *merA* rice plants should aid in stabilizing the soil, enhancing biological activity in these hotspots, and intercepting free Hg(II) before it can be converted to MeHg. Non-engineered plants may avoid these toxic hotspots and thus contribute less to improving soil quality and mercury processing.

merA rice plants grown on mercury-contaminated substrates effectively convert Hg(II) to Hg(0). Ionic mercury added to a hydroponics medium containing one-month old rice plants was bound rapidly to wild-type or *merA* roots, leaving the medium itself virtually devoid of Hg(II). Therefore, the significantly lower concentration of Hg(II) in

the tissues of *merA* rice compared to the wild-type shows that more mercury had been volatilized from the *merA* plants. Significantly lower concentrations of mercury in the aboveground tissues than in roots of wild-type is expected, because the chemically immobile Hg(II) is not efficiently transported through plants [14]. *merA* plants have even lower levels of mercury in aboveground tissues than the wild-type. We speculate that although much of the more mobile and volatile Hg(0) produced by MerA is likely transported through the plant, Hg(0) remains in the aboveground tissues only temporarily before being eliminated from leaves by volatilization and/or transpiration. In this case, the loss of Hg(0) from leaves may parallel the normal stomatal loss of O_2 and H_2O vapor.

Hg(II) cannot bind to plant roots in sediments as rapidly as in hydroponics systems because of the high affinity of Hg(II) for organic and inorganic ligands in the sediment. This high affinity for soils and the heterogenous nature of soil-bound Hg(II), even in laboratory homogenized soils, makes it difficult to compare the mercury processing potential of *merA* and wild-type plants by measuring changes in soil mercury concentration over relatively short periods of time. However, drawing on results from the Hg(II) hydroponics assays, it can be reasonably assumed that a significantly lower concentration of Hg(II) in *merA* rice tissues than the wild-type is a result of higher volatilization of Hg(0) by the *merA* plants. The only alternative is that *merA* plants have a lower capability for absorbing mercury than the wild-type. However it seems unlikely the *merA* gene or MerA protein should negatively impact mercury uptake capacity. Furthermore, no evidence for reduced mercury uptake by *merA* plants is offered by the hydroponics experiments with rice or by the earlier Hg(0) volatilization experiments with other *merA* species such as *L. tulipifera* and *Arabidopsis* [18, 19].

In the field, *merA* rice would likely be planted in soils that have been contaminated for a significant period of time. It has been demonstrated that soon after mercurials are discharged to soils, initial rates of Hg(0) volatilization are very high due to chemical [33] and microbial processes [34, 35]. However, after the most initially available Hg(II) is evolved from the soil as Hg(0), the remaining Hg(II) is tightly bound and highly recalcitrant to extraction. Bacterial remediation is not likely to mediate the cleanup of this more recalcitrant Hg(II), because bacteria provide no means of transporting mercury out of the soil and most Hg(II)-reducing bacteria only live in a narrow depth habitat. In contrast, plants with deep, dense roots and acidic root exudates could extract, detoxify, and remove the mercury.

The mercury-contaminated soils used in these experiment were expected to be relatively recalcitrant to Hg(II) extraction because the laboratory-spiked soil had been contaminated for 3 years and the Oak Ridge soil had been contaminated for about 45 years. The contaminated stream sediment from Oak Ridge, TN has an Hg(II) concentration that is 20 times greater than the laboratory spiked soil. However, it has been exposed to mercury 15 times longer and contains 4 times more sulfur and 2 times more organic matter (Table 1). The greater contaminant age and higher sulfur and organic matter of the Oak Ridge soil were anticipated to result in the Hg(II) remaining less bioavailable than in the laboratory-spiked soil.

The levels of mercury in rice grown in these soils are consistent with differences in soil mercury content, soil composition, and plant genotype. In both the spiked soil and the Oak Ridge soil the *merA* plants processed more Hg(II) from roots and shoots than the wild-type. This difference was most significant for plants grown in the Oak Ridge soil.

Root mercury concentrations were much higher for plants grown in the Oak Ridge soil than laboratory-spiked soil, likely because of the immediate contact of these roots with more highly contaminated soil. Root mercury concentrations in wild-type and *merA* plants grown on the Oak Ridge soil were 45% and 30% of the soil mercury concentration, respectively. However, roots from plants of both genotypes grown on the more recently contaminated laboratory-spiked soil achieved levels of mercury equal to or above that of the soil, showing potential for plants to concentrate mercury from soils. This higher root-to-soil mercury ratio is consistent with the predicted greater bioavailability of the mercury in the spiked soil, compared to in the Oak Ridge soil. In further support of this Hg(II) bioavailability difference, the aboveground tissues of plants showed only a two-fold higher level of mercury on Oak Ridge soils compared to spiked soils despite the 20-fold difference in soil mercury concentrations.

It is likely that if these *merA* plants were grown in a contaminated sediment for a longer period of time they could lower Hg(II) levels in that sediment significantly enough that it could be measured by direct soil analysis. Additional biotechnical approaches could be exploited to accelerate mercurial mobilization and extraction from soils. For example, *merA* plants further engineered to mobilize metals from the soil by excreting larger-than-typical volumes of organic acids from roots could facilitate greater bioavailability of Hg(II) in sediments. Alternatively, because MeHg is less tightly bound to the soil colloid than Hg(II), perhaps plants engineered to express the *merB* gene, capable of converting MeHg to Hg(II), in combination with the *merA* gene, could absorb and degrade MeHg, releasing much less toxic Hg(0). *merB* has been expressed in *Arabidopsis thaliana* in which it has been shown to be effective at converting MeHg to

Hg(II) and then to Hg(0) if coupled with the *merA* gene [16]. The work presented herein suggests that there is significant potential for creating transgenic rice for effective mercury phytoremediation but that further modifications and trials are warranted to maximize the efficiency of the system.

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Table 1. Soil characteristics likely to affect the quantity of Hg(II) available to plant roots

Soil	[Hg(II)] (ppm)	% sulfur	% OM	Years contam.
Cecil, Watkinsville, GA	34.1 ± 4.1	0.015	2.28	3
stream sediment, Oak Ridge, TN	1064.5 ± 61.0	0.058	5.56	~40-50

The Cecil was collected from a farm in Watkinsville, GA and spiked with Hg(II) (see Materials and Methods). The sediment was collected from a stream bank in Oak Ridge, TN. The sediment has a higher percentage than the Cecil of two Hg(II)-immobilizing components, sulfur and OM (organic matter), and had been contaminated for a longer period of time.

Figure 5.1. Hygromycin and mercury selection of *ubi-merA* transformed rice shoots and plants regenerated from embyrogenic calli. A. Transformed calli were placed on shooting medium with 30 mg/l hygromycin. Shoots were formed from transformed tissue within 3 weeks. **B.** Wild-type shoots (left) died in less than 2 weeks when transplanted from MS medium to MS medium with 30 mg/l hyg. Hygromycin resistant (hyg^r) / *merA* transformed shoots (right) survived indefinitely on MS medium with 30 mg/l hyg. **C.** Wild-type (left) and *merA*-expressing (right) rice shoots were taken from selection medium and grown on fertile potting soil. In the absence of Hg(II) in growth substrates, *merA* rice grew no better, and possibly slightly less vigorously than the wild-type. **D.** The majority of *merA*-transformed plants of most lines formed viable seeds. **E.** Transgenic *merA* rice seeds germinated and grew on medium with 250 μM $HgCl_2$ (right), while wild-type seeds began to germinate and then died (center). On left are wild-type seeds germinated on plates with no Hg(II).

Figure 5.1

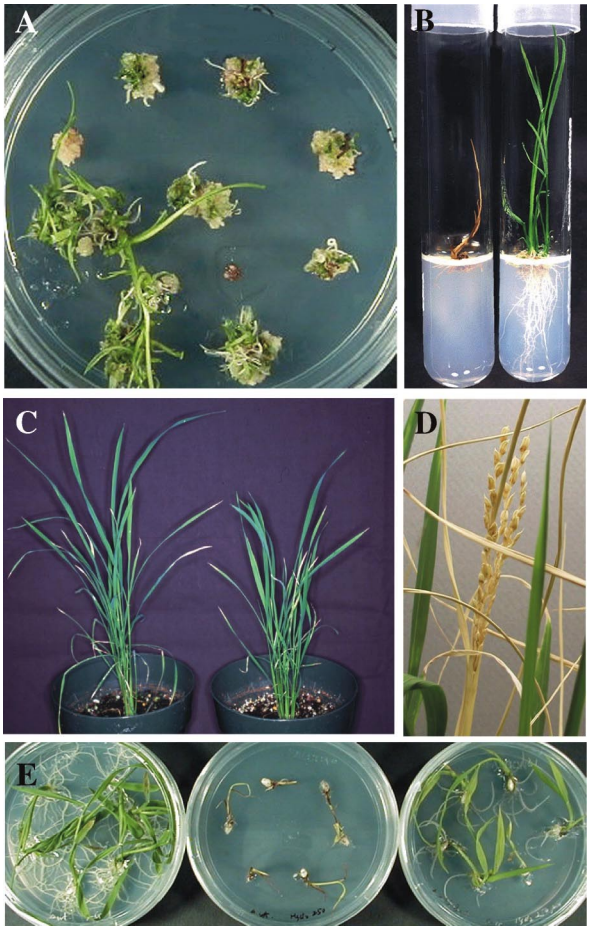


Figure 5.2. Identification of *ubi1-merA* expressing rice lines. A. Several hygromycin resistant rice lines were assayed for the presence of the co-bombarded *merA* DNA sequence using PCR analysis of a plant tissue macerate (see Materials and Methods). The bacterial +*merA* DNA control contained 10 ng of *merA* plasmid. A no-DNA control and DNA extracts from wild-type (wt) rice are also shown. **B**. Several other lines confirmed by PCR to contain the *merA* gene were then assayed for the approximate quality and quantity of MerA protein expresssion using Western blots. Protein extracts from wild-type (wt) rice and *merA*-expressing bacterial controls are also shown as negative and positive controls, respectively. The MerA protein is visible as the double band of about 56 kDa.

Figure 5.2

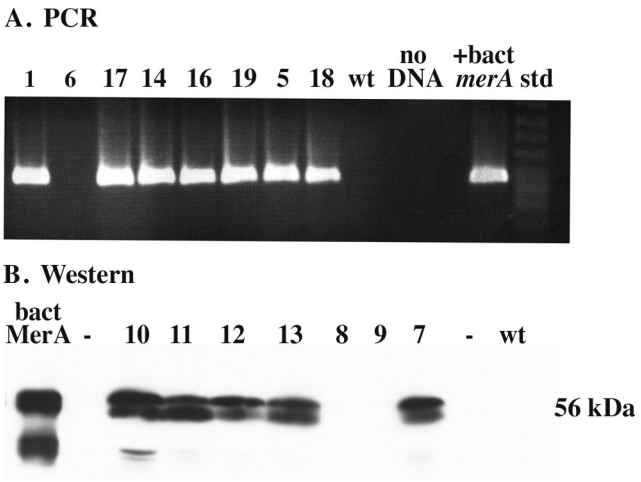

A. PCR
1 6 17 14 16 19 5 18 wt no DNA +bact merA std
B. Western
bact MerA - 10 11 12 13 8 9 7 - wt
56 kDa

Figure 5.3. Transgenic rice seedlings expressing the *merA* gene efficiently volatilized Hg(0). A. Three wild-type rice seedlings and 1 rice seedling from each of 4 *merA* lines were placed individually in a small sidearm test tube in assay medium and $HgCl_2$ was added. Clean air was drawn into and through the reaction tube as each 1-minute sample was taken over a 10-minute assay. The air was bubbled through liquid medium containing a suspended plant sample, and then drawn out of the reaction tube to a mercury vapor analyzer (see Materials and Methods). **B.** Transgenic seedlings segregating from *merA* transformed lines 2, 3, 4, and 15 catalyzed substantial electrochemical reduction of Hg(II) to Hg(0) relative to the reduction seen in 3 control (wt 1, 2 & 3) seedlings. The cumulative amount of Hg(0) volatilized by each time point is shown.

Figure 5.3

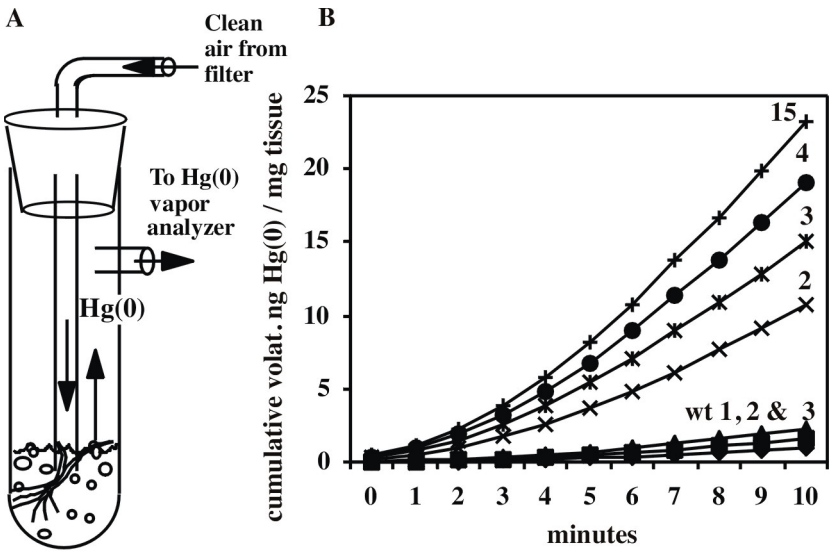
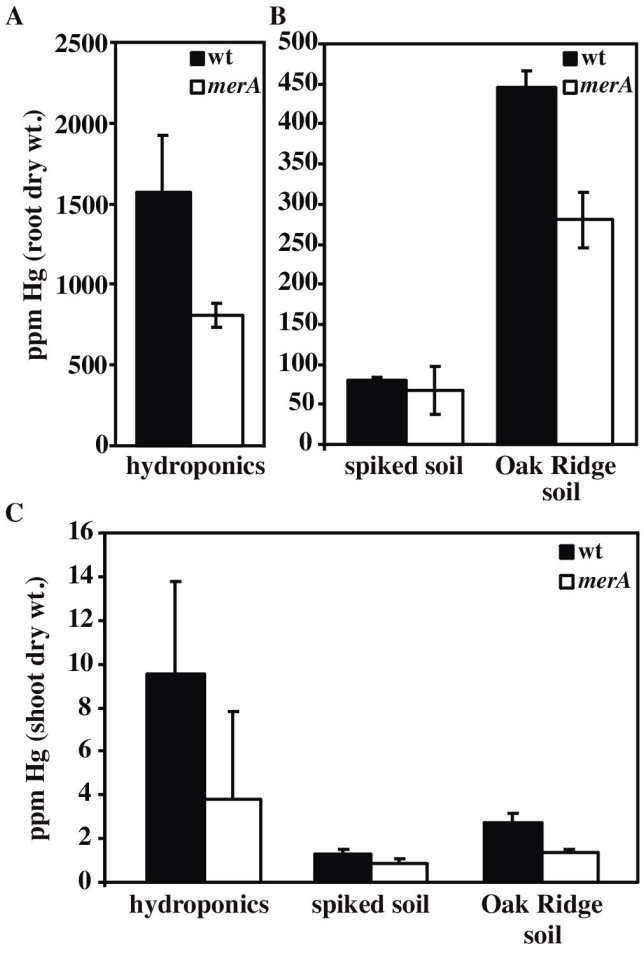

A
Clean
air from
filter
To Hg(0)
vapor
analyzer
Hg(0)
B
cumulative volat. ng Hg(0) / mg tissue
25
20
15
10
5
0
15
4
3
2
wt 1, 2 & 3
0 1 2 3 4 5 6 7 8 9 10
minutes

Figure 5.4. Mercury is processed from mature rice root and shoot tissues expressing the *merA* gene. A. Rice plants from line 15 were grown in hydroponics medium for a 1-week acclimatization period. The medium was then spiked to 2 ppm (10 μM) Hg(II), plants were grown for 10 days, harvested and assayed for the level of total mercury by ICP-MS. Error bars represent 1 standard error for 8 *merA* and 4 wild-type samples. The root mercury concentration in *merA* rice was significantly lower than in wild-type counterparts ($p = 0.07$, t-test). **B.** Rice plants of line 20 were grown in Hg(II)-spiked soil (35.18±2.74 ppm Hg) or field Hg(II)-contaminated stream sediment from Oak Ridge, TN (945.31 ± 28.32 ppm Hg). After 3 months of growth in these soils, plants were harvested, cleaned and analyzed for total mercury (see Materials and Methods). Error bars represent one standard error for 3 *merA* and 4 wild-type samples in the spiked soil and 3 *merA* and 3 wild-type samples in the Oak Ridge soil. The *merA* and wild-type rice were statistically different in root mercury concentration ($p< 0.01$, t-test) for Oak Ridge soils. *merA* and wild-type rice grown in laboratory-spiked soils were not significantly different in root mercury concentration ($p = 0.58$, t-test). **C.** Aboveground tissues from the plants in Figs. 5.4A and 5.4B were analyzed for mercury concentration using the methods employed for roots. Error bars represent one standard error for 7 *merA* and 4 wild-type samples in hydroponics, 3 *merA* and 4 wild-type samples in the spiked soil and 3 *merA* and 3 wild-type samples in the Oak Ridge soils. The *merA* rice were statistically different from the wild-type in shoot mercury concentration for hydroponically grown plants ($p = 0.10$, t-test), for those grown in laboratory-spiked soils ($p=0.15$, t-test) and for those in Oak Ridge soils ($p = 0.02$ t-test).

Figure 5.4


A
B
C
ppm Hg (root dry wt.)
ppm Hg (shoot dry wt.)
wt
merA
hydroponics
spiked soil
Oak Ridge soil
2500
2000
1500
1000
500
0
450
400
350
300
250
200
150
100
50
16
14
12
10
8
6
4
2

CHAPTER 6

SUMMARY AND DISCUSSION

Human industrial activities have moved enormous quantities of mercury from the lithosphere into more mobile biogeochemical pools. In general, this has increased the exposure of organisms and ecosystems to mercury, motivating governments to institute greater controls over the release of mercury. In addition to placing regulations on mercury releases, The United States government has moved to fund studies of mercury pollution. One of these, the US EPA Mercury Study Report to Congress (Keating et al., 1997), has exhaustively detailed the mercury threats posed to public health. The United States government has also funded research projects that seek to find ways to minimize the environmental mercury threat. Research reported in preceding chapters project was funded by the EPA, The Department of Energy and The National Science Foundation because engineering plants for mercury phytoremediation shows promise.

Plants engineered with the *merA* gene show potential as a first step toward developing a strategy for the plant-facilitated remediation (phytoremediation) of mercury polluted soils and water. Initial assays showed that the *merA* gene could be efficiently expressed in plants and that the encoded protein would catalyze the conversion of root-absorbed Hg(II) to Hg(0). However, simple Hg(II) resistance and Hg(0) volatilization experiments were limited to test-tubes or petri-plates with either the very small species

Arabidopsis (Rugh et al., 1996), or with juvenile *Liriodendron tulipifera* (Rugh et al., 1998).

A review of this earlier work was presented in the first half of chapter 2, followed by novel experiments with *merA*-expressing *Nictotiana tabacum* (tobacco). Mature tobacco plants were grown on a hydroponic medium spiked with Hg(II). After the week-long growth period, only very small concentrations of mercury were found in hydroponic medium or in aboveground tissues of *merA* plants or wild-type controls. Of the mercury added, virtually all was bound to plant roots within 24 hours. After 7 days, *merA* plants had volatilized about 70% of this root-bound mercury whereas the wild-type had only volatilized 20%. Such a strong difference between phenotypes suggested that mercury processing of *merA* and wild-type plants could be compared in a growth environment more representative of mercury polluted field sites.

Therefore, *merA* and wild-type tobacco were grown for one month on potting soil containing either no mercury, 100 ppm Hg(II) or 500 ppm Hg(II). On mercury-spiked soils, the Hg(II)-resistant *merA* plants accumulated more biomass than wild-type plants. Total mercury and mercury concentrations in aboveground tissues were lower in *merA* than wild-type plants, likely as a result of *merA*-catalyzed Hg(0) volatilization. Interestingly, mercury was found in aboveground tissues of plants growing on clean soil. Total mercury in aboveground tissues of the wild-type was similar for all soil treatments, suggesting that the majority of total mercury in wild-type tissues may have been absorbed from air as Hg(0). Mercury concentration and total mercury in *merA* plants growing on clean soils were much lower than in *merA* plants growing on mercury-contaminated soils, indicating that most mercury in aboveground tissues was of soil origin. This finding has

important implications for the mercury phytoremediation potential of *merA* plants. In particular, if *merA* plants are capable of moving mercury from roots to shoots before release of volatile Hg(0), they will be more effective at phytovolatilization than if insoluble Hg(0) is forced out of roots back into soil. Furthermore, the low tendency of *merA* plants to absorb Hg(0) in leaf tissues could prevent the recapturing of volatilized Hg(0) during site remediation.

Therefore, a more detailed study of mercury resistance and mercury mobilization in *merA* tobacco was developed (Chapter 3). Mercury resistance of *merA* plants was confirmed by their vigorous growth on semisolid growth medium spiked with concentrations of Hg(II) that were acutely toxic to the wild-type. Mercury resistance allowed *merA* roots to penetrate a barrier of highly concentrated HgS in semisolid growth medium at a much higher frequency than wild-type roots. This suggests that *merA* roots may penetrate mercury "hot spots" in heterogeneously contaminated soils while the wild-type may avoid these areas.

Transpiration measurements of *merA* and wild-type tobacco on hydroponic solution assays demonstrated that plant water movement is less inhibited by mercury exposure in *merA* than in wild-type tobacco. This is probably a result of MerA activity protecting root cells from Hg(II) damage and from the physical Hg(II)-blockage of water transport proteins. The partial alleviation of Hg(II)-induced transpiration stress by *merA* plants may aid in the movement of mercury from roots to aboveground tissues, concurrent with bulk flow of water.

In non-engineered plants, Hg(II) cannot be moved from roots to shoots efficiently because of the high reactivity of Hg(II) and what is thought to be an endogenous plant

enzymatic activity that converts mercury to the oxidized state. In *merA* plants, the *merA* gene should only work against, not replace, such endogenous mercury oxidation. To confirm this, *merA* and wild-type tobacco were grown in a semi-closed chamber containing an elevated concentration of volatilized Hg(0), and the net reductive capacity of leaf tissues was compared by measuring net leaf retention of airborne Hg(0). Because of the specificity of the *merA* gene for Hg(II) only, the difference in net Hg(0) absorption between *merA* and wild-type tobacco likely represented that amount of Hg(0) oxidized to Hg(II) and then re-reduced by *merA* activity. This presents the possibility that even if Hg(0) is insoluble in the transpiration stream, the repeated conversion between the Hg(0) and Hg(II) forms could allow mercury transport from root to shoot.

merA and wild-type plants were reciprocally grafted at the lower shoot to have different root and shoot genotypes, producing a novel tool to further investigate mercury movement through *merA* and wild-type tissues. Tissue analysis of these grafted plants and ungrafted controls grown on Hg(II)-spiked hydroponics medium for 10 days showed that mercury was transported through *merA* tissues more effectively than through wild-type tissues. Plants with wild-type shoots and *merA* roots accumulated more mercury in aboveground tissues than other genotypes or genotype combinations. This suggests that it may be possible to engineer a mercury sequestering plant using the root specific expression of *merA* and the enhanced expression of metal-binding proteins in aboveground tissues.

Success in understanding the effects that *merA* has on expressing plants motivated the introduction of the gene into plant species that would be more practical in field applications than *Arabidopsis thaliana* or *Nicotiana tabacum*. Because mercury pollution

is the greatest environmental threat in soils of mesic habitats or completely submerged sediments where conditions are appropriate for methylmercury formation, species were chosen for their tolerance for waterlogged soils. The *merA* gene was introduced into the dicot *Populus deltoides* (Eastern cottonwood) and the monocot *Oryza sativa* (rice). Both species grow well on flooded soils.

Like previously transformed *merA* species, *merA* cottonwood were highly resistant to root absorbed mercury (Chapter 4). On soils spiked with approximately 420 ppm of Hg(II), *merA* cottonwood survived for 3 months whereas the wild-type died within the first week of the assay. An experiment with lower concentrations of Hg(II) in soils showed that *merA* cottonwood had no significant growth advantage compared to the wildtype until mercury concentrations reached a certain critical point. For that soil, the critical point was between 8 and 20 ppm Hg(II). Aboveground mercury concentrations in cottonwood grown on soils with 0 and 4 ppm Hg(II) were significantly lower than those in the wild-type, and differential net leaf absorption of Hg(0) may have played a major role in these concentrations. The lack of a signficant difference in tissue concentrations between *merA* and wild-type cottonwood on 8 and 20 ppm soils may be explained by the greater pre-harvest loss of mercury-containing leaves from the sensitive wild-type than from *merA* plants.

Rice was the most naturally mercury tolerant plant to be transformed with the *merA* gene, requiring that *merA* plants be screened on Hg(II) concentrations in semi-solid medium of at least 250 μM (Chapter 5). As with other *merA*-transformed species, the high Hg(II) resistance of *merA* rice was associated with a much higher volatilization of Hg(0). Hg(0) volatilization was measured in a reaction bubbler using small rice

germinants immersed in Hg(II) liquid medium. To verify similar activity in mature rice plants, a hydroponic assay was carried almost identically to the tobacco hydroponic assay in Chapter 2. The vast majority of mercury found in plant tissues at the end of the assay was in roots. Significantly lower concentrations of mercury in *merA* than wild-type tissues, particularly in roots, were probably a result of *merA* activity converting Hg(II) to Hg(0), which volatilized from the plant. To more closely simulate the field environment, *merA* and wild-type rice were grown in continuously inundated, Hg(II)-containing soils for 3 months. As with hydroponically Hg(II)-exposed rice, tissue mercury concentrations were much higher in roots than in shoots. As expected, mercury concentrations were lower in *merA* tissues than in the wild-type. The difference was more significant for plants growing in a soil with higher Hg(II) concentration but lower Hg(II) bioavailability. Difficulty in measuring small differences in soil Hg(II) concentration made it impossible to verify greater loss of mercury from soils with *merA* plants.

Indeed, one of the lessons of the work presented in previous chapters is that effective mercury phytoremediation may require a longer period of root / soil contact than the time periods used during these growth chamber assays (≤ 3 months). Hydroponic and bubbler assays have demonstrated that once Hg(II) is made available to the root, *merA* activity will efficiently convert Hg(II) to Hg(0). Therefore it seems that transport from the soil surface to roots may be the limiting factor to the effectiveness of mercury phytoremediation. If *merA* plants are grown for longer periods, the soil in direct contact with roots may become depleted in nutrients and roots may grow into the most highly-contaminated microsites in search of higher nutrient concentrations. Furthermore, after roots have infiltrated the soil completely to absorb the most readily-available

nutrients, they may mine for nutrients more strongly, possibly using acidic exudates or organic chelators. Recent work (unpublished so far) has shown that tobacco plants grown at a field site for slightly longer than 3 months in Hg(II)-contaminated soil lowered the concentration of Hg(II) to a greater extent than either the wild-type or other non-engineered plants.

However, it is possible that plants will not carry out highly efficient mercury phytoremediation until they are further engineered with the capacity to mobilize Hg(II) from soil particles. Both plant-excreted and synthetic chelators can mobilize metals such as Hg(II) in soils. It may be possible to engineer increased production of chelators such as mugineic and avenic acids. These chelators and others could be chosen for their specificity for particular metals, such as mercury (Meagher, 2000).

Possibly the most common criticism of the *merA* mercury phytovolatilization project is that Hg(0) volatilization may increase the mobility of mercury which otherwise would be relatively harmless in soils or sediment. However, the year-long residence time of Hg(0) in the atmosphere means that volatilized mercury would enter a global atmospheric pool containing a 5×10^9 Kg Hg(0) (Mason et al., 1994), making only a negligible contribution to this pool, even if Hg(0) phytovolatilization were widespread and highly efficient. On the other hand, even a small quantity of mercury leached or washed into bodies of water can be converted to methylmercury of high enough concentrations to cause measureable harm to organisms.

For example, in the Amazon, it is estimated that as much as 1.5×10^5 Kg of mercury are released into the atmosphere each year by gold-mining activities (Pfeiffer et al., 1993). This huge input of Hg(0) increases the estimated global atmospheric pool by

three thousandths of one percent and likely has only a negligible effect on human or ecosystem health. A much smaller quantity of mercury from the Amazon mining source is released into rivers, streams and riparian soils. As a result, fish in these waters are often severely contaminated with methylmercury, and humans who eat the fish have elevated levels of methylmercury in their systems (Pfeiffer et al., 1993). In this case, even huge amounts of Hg(0) release are likely to have insignificant hazardous effects compared to much smaller releases of mercury into bodies of water, followed by conversion to methylmercury. If any sizeable fraction of this mercury could be intercepted by plant roots and released as Hg(0), it would likely be to the advantage of the humans and ecosystems of the Amazon basin and likely would not be a significant disadvantage to humans and ecosystems throughout the world.

However, we recognize that the location of a highly mercury-contaminated site could invite concern about Hg(0) exposure resulting from phytovolatilization. For example, if a highly mercury contaminated site were located in an urban area, or positioned so that local wind patterns would tend to blow Hg(0) toward a highly populated area, another remediation strategy might seem more appropriate. Therefore, we have been developing a plant that would be capable of moving mercury from soil and sequestering it in aboveground tissues instead of volatilizing Hg(0). Grafting wild-type tobacco shoots to *merA* roots and exposing the grafted plants to highly available Hg(II) in hydroponic medium (chapter 3) suggested that mercury absorbed by roots of these plants could be moved to aboveground tissues and sequestered there.

As the first step toward creating a mercury sequestering plant, we are genetically engineering a plant with *merA* gene expression in root tissues only. The *merA* gene was

cloned with each of two root-specific promoters and introduced into tobacco. Plants with root-specific *merA* expression have a moderate level of Hg(II) resistance that is between the Hg(II) sensitivity of the wild-type and the high Hg(II) resistance of constitutive *merA* plants. Hydroponically exposing these plants to Hg(II) has shown that belowground mercury processing capability is identical to that in constitutively expressed *merA* tobacco. Mercury sequestration in shoots of root-specific *merA* tobacco was significantly higher than in constitutive *merA* shoots but significantly lower than in wild-type shoots. Higher shoot mercury concentrations in root-specific than constitutive *merA* tobacco was expected. However, lower mercury concentrations of *merA* shoots than wild-type shoots was not predicted. It may be attributable to a large loss of Hg(0) directly from roots in root-specific *merA* plants compared to mercury retention and slow vertical translocation in wild-type plants.

Additional genetic modifications to *merA*-expressing plants may be necessary to make them effective at cleaning mercury-contaminated soils, however the *merA* plant technology is a powerful starting point. Enormous strides have been made since the first *merA* plant data were published in 1996. The *merA* gene has been introduced into numerous species including tobacco, eastern cottonwood and rice, all described in the preceeding chapters. *merA* tobacco provided a model plant for the detailed investigation of the effects of *merA* gene in plant mercury interactions and processing. Cottonwood and rice were engineered with the *merA* gene because of their tendency to grow in habitats where mercury pollution is the greatest threat. Experiments with these species in soils suggest that mercury phytoremediation has potential to work but will either require

longer growth periods than 3 months or may require additional genetic engineering to perfect.

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