SCOTT PATRICK BIZILY
Genetic Engineering of Plants with the Bacterial Genes *merA* and *merB* for the Phytoremediation of Methylmercury Contaminated Sediments
(Under the Direction of RICHARD B. MEAGHER)

Methylmercury (CH_3Hg^+) is an environmental toxin that biomagnifies in the aquatic food web with severe consequences for humans and wildlife. To reduce the incidence of CH_3Hg^+, our lab is engineering plants to be used for in situ processing (phytoremediation) of mercury-contaminated sediments. Our ongoing stategy has been to provide plants with the bacterial genes for organomercurial lyase (*merB*) and mercuric reductase (*merA*), two enzymes that operate sequentially to convert CH_3Hg^+ to a less hazardous form of mercury, Hg(0). Elemental mercury is volatile and, therefore, can be transpired through plant stomata and diluted in the atmosphere.

We used the model system, *Arabidopsis thaliana*, to test our proposed strategy. Plants containing *merA* and *merB* were shown to grow vigorously on levels of methylmercury and phenylmercuric acetate (an alternative substrate for MerB) that are lethal to wild-type plants. An in vivo biochemical assay was used to demonstrate that *merA/merB* plants absorb organic mercury from a liquid medium and release Hg(0). We also showed that the rate of organic mercury degradation is related to the steady-state concentration of MerB and that this enzyme localizes to the cytoplasm. However, variations in combined MerA and MerB enzyme expression explain only ~40% of the variation in the rate of organic mercury degradation. This suggested that the diffusion of the organic mercury substrate to MerB is inefficient and that organic mercury may be accumulating at the cell wall or in membrane-rich compartments away from the enzyme.

A final research objective has been to determine whether the efficiency of organic mercury detoxification can be improved by altering the distribution of MerB.

Specifically, we have created new gene constructs containing signal sequences to target MerB to the cell wall and the endoplasmic reticulum. After transforming plants, we analyzed the T_2 generation for organic mercury resistance, enzyme expression levels, and rates of organic mercury conversion. Plants in which MerB has been targeted to the secretory pathway appear to be much more efficient at degrading organic mercury than plants that express MerB cytoplasmically. With approximately 10-100X less protein, they achieve resistance levels and rates of degradation comparable to plants with the wild-type *merB* sequence.

INDEX WORDS: *Arabidopsis thaliana*, bioremediation, environmental pollution, MerA, MerB, mercury pollution, Minamata disease

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by

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Methylmercury (CH_3Hg^+), the methylated derivative of ionic mercury [Hg(II)], is considered one of the world's most serious environmental pollutants. Although its presence at contaminated sites may be at the low range of detectability, methylmercury can biomagnify in marine wetland ecosystems to levels sufficient to harm fish and their predators. At sublethal concentrations, methylmercury acts as a neurotoxin in humans, causing degradation of the CNS and associated defects in cognitive performance. Since the late 1960's, following a large epidemic of methylmercury poisoning in Minamata Bay, Japan (Harada 1995), numerous studies have explored the mechanisms of methylmercury toxicity in humans and other organisms, the presence of methylmercury in the environment, and the biological and non-biological transformations of methylmercury and other mercurials. The overwhelming conclusion from these studies is that it is critically important to prevent the release of mercurials into the environment. Due to the high cost of traditional cleanup procedures such as excavation, dredging, and landfilling, as well as the difficulty of assigning fiscal responsibility, most contaminated sites have not yet been remediated. Our research is an attempt to find a more cost-effective and environmentally-responsible method for remediating mercury contaminated soils and sediments. Our strategy utilizes plants to absorb and detoxify mercury pollutants and therefore can be considered part of the broader field of phytoremediation.

Mercury and mercurial compounds are hazardous to nearly all biological organisms and have been shown to interfere with a wide range of physiological processes. Though mercury is typically found at trace levels in the earth's crust, anthropogenic activities have created areas with elevated and potentially dangerous

concentrations (Keating *et al*. 1997; Nriagu 1979a). Bacteria have evolved at least one mechanism for eliminating mercurials and preventing cellular damage, a contiguous series of mercury resistance (*mer*) genes encoding transporters and enzymes for biochemical detoxification. *Mer*$^{+}$ bacteria convert organic and ionic mercury compounds to the volatile and less toxic elemental form, Hg(0), which rapidly evaporates through cell membranes. By genetically engineering plants with two of the *mer* genes (*merA, merB*), we hoped to recapitulate the biochemical detoxification pathway and enable the plants to move concentrated mercury from the ground into aerial plant tissues and/or into the dilute atmospheric pool. Presently, we have tested these genes in two plant species, *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*), and are preparing to test them in species that are more suitable for field conditions (e.g. rice, cottonwood, marsh grass). We have demonstrated that transgenic plants grow in the presence of typically lethal levels of ionic and organic mercury. Furthermore, these plants enzymatically reduce mercurial compounds extracted from spiked growth media and transpire elemental mercury (Bizily *et al*. 2000; Bizily *et al*. 1999; Rugh *et al*. 1996).

While we are optimistic that phytovolatilization will prove to be an effective field technique for removing mercury, we also appreciate the broader implication of our work. Genetic engineering will provide a powerful tool for adapting plants to new and wide-ranging roles in environmental restoration. In particular, it will be feasible to engineer a variety of species that are native to specific terrestrial, wetland, and aquatic habitats for phytoremediation. By combining sets of genes that confer activity towards different pollutants, it may also be possible to develop plants that detoxify a broad range of chemicals.

Atmospheric Emission of Mercury

Mercury emission rates have risen from pre-industrial levels of ~1.6 x 10^6 kg/yr to current emissions by natural and manmade sources of 4-6 x 10^6 kg/yr (Keating *et al*. 1997). Increased emissions have elevated the global atmospheric pool from ~2 x 10^6 kg to ~4 x 10^6 kg mercury, primarily as Hg(0), over the last century (Slemr and Langer 1992). Smoke-stack emissions of mercury occur in three principle forms: free Hg(II), particle-bound Hg(II), and Hg(0). Free and particle-bound Hg(II) have atmospheric residence times on the order of weeks with ~70% of emissions deposited near their point source (Keating *et al*. 1997). By comparison, Hg(0) atoms are retained ~0.5-2.0 years in the global atmospheric pool (Lindqvist 1994; Lindqvist and Rodhe 1985). Although mercury deposition rates have declined in parallel with patterns of mercury use over the last three decades (Benoit *et al*. 1994; Swain *et al*. 1992), the global atmospheric Hg(0) pool has increased in magnitude. This apparent inconsistency may be explained by significant declines in overall particulate matter pollution and a concordant reduction in Hg_p formation and deposition (Hudson *et al*. 1995). The principal sources of atmospheric mercury emission in the USA are waste incinerators and fossil fuel burners and engines, which collectively account for >80% of all anthropogenic sources (Keating *et al*. 1997). In order to achieve a 90-95% reduction in U.S. emissions from these sources, the US Environmental Protection Agency is issuing recommendations for separating out mercury-containing medical and municipal waste before incinerating bulk wastes and for applying carbon-based treatment systems (Keating *et al*. 1997).

Terrestrial and Aquatic Release

Mercury and mercurial compounds have been used in a variety of consumer and agricultural products such as paints, crop sprays, and dental amalgams. Several industrial processes use mercury or mercurial compounds: chlor-alkali synthesis (used in electrodes), wood pulping (as a fungicide), precious metal extraction (to form amalgams). One of mankind's most notorious environmental mishaps was the contamination of Minamata Bay, Japan in the 1950's and 1960's by run-off from an acetylaldehyde plant (Harada 1995; Takizawa 1979). This prolonged and unintentional release of organic mercury claimed more than 1000 lives and caused irreversible neurological damage in 5000-6000 people, all of whom were exposed by consuming contaminated seafood (Harada 1995). The Minamata tragedy helped to curtail the extensive industrial use of mercury and motivated governmental agencies to enact strict controls on its disposal. Due to the increased documentation of the health hazards of mercury exposure, most consumer use also has been discontinued and manufacturers have replaced mercurial compounds with alternative agents or processes (Nriagu 1979b).

The use of mercury to extract gold from ore remains a significant source of environmental contamination in economically and technologically underdeveloped countries as well as some American states (Akagi *et al*. 1995; Keating *et al*. 1997; Pfeiffer *et al*. 1993). The gold mining industry in the Brazilian Amazon, for example, consumed an average of 137 tons of mercury per year during the 1980's (Ferreira and Appel 1990). Based upon averages for gold production and emissions factors of 1.3-2.0 kg Hg released/kg Au produced, Pfeiffer et al. (Pfeiffer *et al*. 1993) estimate that 130 tons Hg/yr. were released into the Amazon forests. Most of this sum (65-83%) would have

been emitted into the atmosphere during the roasting of the Hg/Au amalgam with the rest spilling into soils and waterways (Pfeiffer *et al*. 1993). Soils and sediments near many of the Brazilian mining areas have been monitored by scientists and the levels of contamination reported range as high as 19.8 ppm (Pfeiffer *et al*. 1993). This is a relatively low figure when measured against data for many American sites contaminated by run-offs from nuclear and chlor-alkali plants. However, the fact that the pollution in the Amazon is dispersed over an enormous area should not permit us to become less vigilant. As anthropogenic mercury deposits in and around waterways increase in magnitude, mercury may eventually reach hazardous concentrations in fish and other wildlife.

Mercury Biogeochemistry

Ionic mercury (II) is the prevalent terrestrial species and is found associated with a number of different anions, such as chlorides, sulfides, organics, and hydroxides (Anderson 1979). Hg(II) compounds undergo various chemical transformations by abiotic (Allard and Arsenie 1991) or bacterial processes (Robinson and Tuovinen 1984). Abiotic and microbial reduction of Hg(II) from Hg-containing sites generates 25-50% of global atmospheric Hg(0) emissions (Fitzgerald 1995; Keating *et al*. 1997). *In vitro* and *in situ* analyses have demonstrated that abiotic reduction of ionic mercury by humic and fulvic acids releases Hg(0) vapor (Allard and Arsenie 1991). Sunlight appears to promote humic acid-associated Hg(II) reduction (Xiao *et al*. 1995).

Some bacteria colonize mercury-contaminated habitats and are capable of enzymatically detoxifying Hg(II) and organomercurial compounds (Robinson and

Tuovinen 1984; Summers and Lewis 1973). These characteristics are conferred by the *mer* operon, a series of genes which encodes two regulatory proteins, two mercury transport peptides, and, for broad spectrum (organomercurial) mercury-resistance, two catalytic peptides (Summers 1986). Broad spectrum-resistant bacteria express the enzyme organomercurial lyase (MerB) (Begley *et al*. 1986a; Begley *et al*. 1986b; Tezuka and Tonomura 1976) which recognizes several forms of organic mercury and catalyzes the protonolytic cleavage of the carbon-mercury bond. This reaction provides the inorganic substrate [Hg(II)] for MerA (mercuric reductase), an NADPH-requiring, FAD-containing mercuric ion oxidoreductase that catalyzes the electrochemical reduction of Hg(II) to Hg(0) (Fox and Walsh 1982). Since elemental mercury is volatile and evaporates, bacteria are able to eliminate their mercurial load without having to expend energy pumping it out of cytoplasm.

Sulfate-reducing bacteria in anaerobic sediments methylate Hg(II), accounting for at least 90% of environmental methylmercury (MeHg) formation (Compeau and Bartha 1985). Bacterial methylmercury synthesis has been observed to occur more efficiently under acidic, saline, and anoxic conditions (Beijer and Jernelov 1979; Ridley *et al*. 1977). Aerobic bacteria also produce methylmercury, though at much lower rates and efficiencies than sulfate-reducing bacteria (Olson and Cooper 1976).

Mercury Toxicity

Mercury and mercury compounds are non-nutrient poisons that cause severe physiological effects when ingested in relatively small doses (μg quantities/day). The primary mechanism of toxicity is thought to be the reaction by charged forms of mercury

with reduced sulfhydryl groups and imino-nitrogen groups on critical cellular proteins. The binding of mercury to these macromolecules inhibits their normal biological functions and disrupts cellular metabolism. The most noteworthy symptoms of mercury poisoning include tremors, sensory disturbances, ataxia, and parathesia. Histological studies have confirmed that mercury poisoning can lead to the atrophy of the central nervous system.

The toxicokinetics of the three principal forms of mercury, Hg(0), Hg(II), and methylmercury (MeHg), are substantially different. Elemental mercury is readily absorbed through the lungs and passively diffuses through other biological tissues yet has a relatively short residence time. It does not accumulate nor react with most biochemicals unless first oxidized to Hg(II) by peroxidase-catalase enzymes (Ogata and Aikoh 1984). Victims who experience either chronic or intense, short-term exposure may experience neurological dysfunction. Only a few deaths have been attributed to Hg(0) inhalation which in all reported cases resulted in respiratory failure.

Despite its high chemical reactivity, Hg(II) does not freely diffuse through cellular membranes and is inefficiently absorbed through the intestines. Studies have reported that ~80-85% of ingested doses are excreted in feces (Miettinen 1973) with the rest being eliminated over a period of days or months through urine (Rahola 1973). As Hg(II) is processed and filtered by the body, it is likely to cause extensive damage to the kidneys, liver, and gastrointestinal tract. These organs will typically be affected at much lower doses than are required for the onset of neurological degeneration.

Methylmercury (MeHg) is lethal at much lower doses than Hg(0) or Hg(II) because it is both reactive yet highly mobile. It is efficiently absorbed from the

gastrointestinal tract with an estimated uptake of 95% in humans (Aberg 1969; Miettinen 1973). Once inside the circulatory system, MeHg is rapidly distributed throughout the body. Unlike Hg(II), it is readily transported across blood vessel walls and into tissues. The membrane permeability of MeHg has been variously attributed to its hydrophobic character, to its tendency to associate with counterions (e.g. Cl^-) to form neutral, weakly dissociating salts (Mason *et al*. 1996), and to the recognition of a cysteine-bound MeHg complex by a transporter protein (Aschner and Aschner 1990; Kerper *et al*. 1992; Mokrzan *et al*. 1995). Though it may have a widespread impact on many basic cellular processes, MeHg poisoning is typically identified by abberant neurological responses. MeHg has been shown to interfere with acetylcholine neurotransmitter release by disrupting the flow of Ca^{2+} through ion channels (Atchison and Hare 1994). Initial symptoms of poisoning may include shaking, difficulty in walking, and other sensory impairments. At greater durations and/or magnitudes of exposure, victims experience memory loss, sensory loss, and an inability to walk in parallel with a rapid deterioration of the CNS. The syndrome caused by MeHg poisoning has been termed Minamata disease following the outbreak at Minamata Bay, Japan. Because MeHg has a long residence time and is efficiently transferred between trophic levels it is biomagnified through the food web (Jernelov and Lann 1971). Bioconcentration factors ($B_f = \log [CH_3Hg^+_{biota}] / [CH_3Hg^+ H_2O]$) have been estimated at 5-6 units for fish indicating that parts per trillion levels in waters can translate into ppm levels in fish (Watras and Bloom 1992). For habitual consumers of fish, these tissue concentrations are often sufficient to cause neurotoxicity. Based upon the available toxicity data, the U.S. EPA has set a reference dose of 0.1 μg/kg/day for human consumption (Keating *et al*. 1997).

Non-Biological Mercury Remediation

As with most xenobiotic pollution, mercury contamination is difficult and expensive to remediate. The restoration of heavy metal contaminated areas typically involves the relocation or intensive on-site treatment of large quantities of soils and sediments. These techniques are often extremely disruptive to the endogenous wildlife and may delay the full ecological recovery of polluted sites. However, for severely contaminated environments that require immediate action, the best option may be to transfer polluted soils to lined landfills.

The bulk relocation of mercury-contaminated soils and sediments is the most common method for the remediation of polluted sites. High cost, modest efficiency, and incidental Hg mobilization are drawbacks to this approach (Schultz *et al*. 1995). In recent years, new technologies have minimized the loss of mercury from contaminated sediments during dredging operations and simultaneously raised the efficiency of pollutant recovery (Herbich 1995). Improvements include the use of hydraulic suction dredgers to avoid violent mechanical "cutting" into sediment floors and the addition of camera monitoring systems to measure turbidity and permit the control of suction force. Both of these dredging strategies were employed to reclaim the contaminated Minamata Bay area (Yoshinaga 1995). During this operation, sediment resuspension was carefully controlled as greater than 1.5 million cubic meters of mercury-containing sediments were moved onto the innermost 580,000 square meters of the site over a ten-year period. The sediment mounds were enclosed in a constructed watertight revetment, overlaid with plastic, and then capped with clean soil. Using these advanced techniques, the hazards of dredging can be mitigated, though the cost remains prohibitively high for most sites.

Dredged, excavated waste is usually relocated, incurring the expenses of transportation and landfill burial. In addition, organic leachates and anaerobic conditions found in landfills have been demonstrated to promote Hg(II) mobilization and methylation (Neibla *et al*. 1976).

To prevent the escape of mercury and other heavy metal toxins, cements or resin polymers may be added to solidify contaminated sludge material. Waste solidification reduces volume and water content and prevents the leaching of most hazardous materials. However, mercury is more prone to leaching than other chemicals and may not be adequately contained by thermal solidification treatments or low temperature stabilization. Complexing of the ionic mercury with sulfides prior to treatment may help to minimize this problem (Hamilton and Bowers 1997). Another approach to removing mercury waste utilizes an electrical apparatus to heat soil and evaporate the contaminant. This process is expensive and destroys the immediate utility of the vitrified soil, but may be suitable for urgent, small-scale spills.

Mercury Bioremediation

Bioremediation is the use of living bacteria or immobilized biomass for the treatment and clean-up of hazardous wastes. Toxin-degrading organisms may be added to waste sites (bioaugmentation) or amplified by enriching the substrate with essential nutrients (biostimulation) (Bandyopadhyay *et al*. 1994). Alternatively, mats of living cells or dead biological material may be used to absorb and filter pollutants from effluent streams. Some bacteria enzymatically reduce particular heavy metals to insoluble precipitates as a means of detoxifying them (Silver and Phung 1996). When added to

wastes these bacterial strains may help to make heavy metals more recoverable (Rawlings and Silver 1995).

In light of their ability to detoxify and volatilize mercury, *mer*$^{+}$ bacteria are obvious candidates for bioremediation, and they have already been used in trial experiments. *Pseudomonas* strain K-62 was shown to reduce mercurial compounds to Hg(0) from industrial wastes (Suzuki *et al*. 1968), although its rate of volatilization was sharply affected by suboptimal salt concentrations. Similarly, Hansen *et al.* (Hansen *et al*. 1984) treated mercury-contaminated sewage with a continuous culture of mercury-resistant bacteria and observed that the bacteria removed 98% of Hg(II) by converting it to Hg(0). Given that bacteria are extremely sensitive to environmental conditions and are difficult to manipulate in the field, their most promising role as bioremediators will probably be in controlled industrial applications.

Biosorption is the use of immobilized cells or biomaterials to nonspecifically bind and remove reactive and particulate heavy metals from waste streams (White *et al*. 1997). The strategy has been applied to mercury and other contaminants. For example, Wilkinson *et al.* (Wilkinson *et al*. 1989) used immobilized algal cultures to accumulate Hg(II) and found that algae filtered out more than 95% of the total mercury treatment from the medium. Unfortunately, biosorption reactor flow rates and volumes are usually very limited and will probably restrict this technology to small-scale applications (Gadd and White 1993). In addition, biosorption is sensitive to contaminants such as oils or particulates, making it unsuitable for many field-site cleanup applications (Brierly *et al*. 1989).

Phytoremediation

The term "phytoremediation" refers to the use of plants to extract pollutants from contaminated soils and convert them to less toxic or non-toxic substances (Chagnon *et al*. 2000; Chaney *et al*. 1997; Cunningham and Berti 1993; Cunningham and Ow 1996). The end products are either stored and concentrated in above-ground tissues or volatilized and diluted in the atmosphere (Meagher *et al*. 2000). Phytoremediation is an old concept possibly dating to the use of plant mats to filter local water supplies in Western Europe. The fact that plants were recognized as effective filtering devices without the aid of chemical instrumentation for analyzing water quality, underscores their ability to actively uptake and/or non-specifically bind a variety of metallic and organic pollutants (Meagher 2000).

Renewed interest in phytoremediation, both within the scientific and environmental engineering communities, emerged in the 1960's as plant biologists began to study mechanisms of metal resistance in plants. More recently, scientists have come to appreciate that species differ widely in their responses to specific pollutants and have different strategies for tolerating them. Some plants, termed hyperaccumulators (Baker 2000; Baker and Brooks 1989; Raskin *et al*. 1994; Reeves and Baker 2000), concentrate metals in vacuoles or lignin, preventing them from interferring with vulnerable intracellular components. Natural hyperaccumulation may, in fact, be an evolved approach for warding off insects, considering that most plant species take up toxic metal ions inefficiently. As defined by the scientific community, hyperaccumulation is the concentration and tolerance of >1% shoot dry biomass of a given element or chemical compound. As one might expect, hyperaccumulators concentrate specific metal ions that

are abundant in the soils in which they grow. Zn (Ebbs *et al*. 1997; Lasat *et al*. 2000; Shen *et al*. 1997), Ni (Anderson *et al*. 1999; Robinson *et al*. 1997a; Robinson *et al*. 1997b), and Se (Banuelos *et al*. 1997; Banuelos *et al*. 1998; Pilon-Smits *et al*. 1998) hyperaccumators have been studied in the lab.

Unfortunately, hyperaccumulators for the most dangerous metals, Cd, As, Ur, and Pb, have not been found in nature. Modern molecular biology, however, may offer the means to convert modest accumulators into efficient tools for phytoremediation. *Thlaspi caerulescens* (alpine pennycress), for example, has been found to accumulate up to .1% Cd at contaminated sites making it, at least, a valid study subject (Reeves and Baker 2000). *Amaranthus blitoides* (prostrate pigweed), *Brassica Juncea* (Indian mustard), and *Pteris vittatta* (brake fern) are candidate species for arsenic remediation though will probably require extensive genetic modification before they can be used as commercial arsenic processors (deHaro *et al*. 2000; Ma *et al*. 2001; Pickering *et al*. 2000). Indian mustard plants have also been shown to concentrate Pb to .01-.06% of s.d.b. (Huang *et al*. 1997), making them one of the highest Pb-accumulating species found thus far. The addition of EDTA to soils causes Indian mustard to accumulate >1% s.d.b. of Pb as an EDTA-Pb chelate (Cooper *et al*. 1999; Epstein *et al*. 1999; Huang *et al*. 1997), suggesting that lead phytoremediation may be feasible in the presence of the right soil amendments. Similarly, uranium uptake by plants grown on contaminated soil is initially low but increases dramatically (~1000X) upon the addition of the chelator, citrate. *Brassica juncea* and *Brassica chinensis* can thus be induced to accumulate up to .5% uranium (Huang *et al*. 1998). In addition to citrate, other naturally-produced chelators such as histidine, asparagine, and nicotianamine also facilitate metal uptake. These chelators are

synthesized by plant biochemical pathways and exuded by plant roots into the soil. A variety of labs are attempting to increase the synthesis and release of chemical chelators by genetically modifying relevant pathways. One lab has already shown that aluminum tolerance can be improved by genetically enhancing citrate synthesis (de la Fuente *et al*. 1997).

The mechanisms by which plants tolerate organic pollutants differ from those for metal pollutants in that they are based on biochemical decomposition rather than sequestration. Plants naturally produce a range of enzymes including laccases, dehalogenases, nitroreductases, nitrilases, and peroxidases that recognize organic chemicals. Elevated expression in particular species promotes the degradation of organic contaminants such as TNT (Bhadra *et al*. 1999a; Bhadra *et al*. 1999b; Hughes *et al*. 1997; Larson *et al*. 1999; Lucero *et al*. 1999; Pavlostathis *et al*. 1998; Scheidemann *et al*. 1998), petroleum (Nedunuri *et al*. 2000; Wiltse *et al*. 1998), atrazine (Burken and Schnoor 1996; Burken and Schnoor 1997), PCB's (Kucerova *et al*. 1999; Mackova *et al*. 1997), and phenanthrene (Liste and Alexander 1999). Optimally, the contaminants are broken down into volatile metabolites such as H_2O, CO_2, and NO_2. In many cases, however, primary contaminants are converted to intermediates that are still toxic. Again, researchers are using genetic engineering to create plants with improved degradative capacity (French *et al*. 1999; Macek *et al*. 2000).

Two other classes of genetic modification are being tested, or at least discussed, for their abilities to enhance phytoremediaton. First, membrane transporters are being isolated and characterized for their abilities to assist in the uptake of specific solutes from groundwater (Guerinot 2000). Some transporters recognize toxic metal ions in addition

to nutrient ions. By increasing the expression of specific transporters, scientists may be able to promote hyperaccumulation. Because high intracellular concentrations of heavy metals can be toxic to plants, scientists also need to look at ways to enhance tolerance mechanisms. A final class of genetic modifications would take advantage of natural metal-binding peptides such as metallothioneins (MT's) (Goldsbrough 1998; Hamer 1986; Kagi and Kojima 1987; Kägi and Schaffer 1988; Robinson *et al*. 1993; Tomsett *et al*. 1989) and phytochelatins (PC's) (Clemens *et al*. 1999; Cobbett 1999; Goldsbrough 1998; Grill *et al*. 1985; Rauser 1990). MT's are encoded proteins of approximately 60 - 80 amino acids with 9 - 16 cysteines at stragetic locations. The cysteines are folded together to form metal-coordination complexes. PC's also bind metals using similar cysteine-based coordination complexes. PC's, however, are biochemically synthesized from the amimo acid substrates, glutamate and cysteine. The pathway involves three enzymes, gamma glutamyl cysteine synthetase, glutathione synthase, and phytochelatin synthase. Molecules of approximately 2-3 KDa are produced. Together, MT's and PC's are manufactured in response to high intracellular metal concentrations, can act as sinks for metal ions, and help mediate metal resistance. In certain cases, MT-metal and PC-metal complexes are recognized by vacuolar transporters and thereby play a part in vacuolar deposition. A variety of phytoremediation strategies are based on using genetic modifications to overproduce PC's and MT's (Evans *et al*. 1992; Hasegawa *et al*. 1997; Yeargan *et al*. 1992; Zhu *et al*. 1999).

Plant Interactions with Mercury

Plants are capable of extracting a variety of metal ions from their growth substrates, including mercury. Field and laboratory experiments on plant uptake and phytotoxicity have demonstrated that plants accumulate mercury from both soil and atmospheric sources (Godbold 1991; Godbold and Huttenmann 1986; Lindberg *et al*. 1979). The rate of uptake depends largely on the organic content, the pH, and other characteristics of the growth medium (Adriano 1986).

Since mercury is not an essential mineral, plants have not evolved special mechanisms to transport the heavy metal from roots into aerial portions of the plant. Because plants exhibit a variety of vascular morphologies, the efficiencies at which they move mercury through xylem is likely to vary between species. In most cases the rate of transport will be much lower than those for nutrient metal ions such as Zn(II) and Fe(III). Based upon its high mobility in other biological systems, organic mercury may move into plant shoots faster than Hg(II).

At sites with high atmospheric mercury loads, mercury is detectable in the foliage of local vegetation (Huckabee *et al*. 1983). Although some of the foliar content may be due to root uptake and subsequent transport, most of it is probably directly deposited into leaves as Hg(0) (Lindberg *et al*. 1986). Plants exposed to foliar Hg(0) treatments do not accumulate mercury in their roots despite the high mobility of elemental mercury, indicating that downward transport via phloem is negliable (Suszcynsky and Shann 1995). The oxidation of elemental mercury vapor to less mobile Hg(II) by leaf catalases may serve to restrict Hg(0) translocation. Plant transpiration has been observed to significantly elevate Hg(0) evolution from mercury-containing soils (Hanson *et al*. 1995;

Kozuchowski and Johnson 1978). This observation suggests that plants naturally reduce Hg(II) at a low, background level. By absorbing mercury from both the ground and the atmosphere, plants play a dynamic role in the cycling of Hg(0) at the soil-air interface (Lindberg *et al*. 1986; Siegel and Siegel 1979).

Plants tolerate moderate levels of inorganic mercury in the soil and atmosphere though may grow more slowly than in "clean" environments. When challenged with toxic levels of mercury they display severely stunted growth, leaf curling, and chlorosis. Mercury is known to affect photosynthesis and oxidative metabolism by interfering with electron transport chains (Siegel *et al*. 1974). It also diminishes the uptake of water (Beauford *et al*. 1977) and the homeostasis of nutrients and electrolytes by altering membrane potentials and inhibiting ion channels and pumps (Godbold 1991; Kennedy and Gonsalves 1987; Kennedy and Gonsalves 1989). While organic and inorganic mercury seem to damage plants via the same basic mechanisms, compounds such as phenylmercuric acetate and methylmercury chloride appear to be 2-4 orders of magnitude more toxic than Hg(II) (Bizily *et al*. 1999; Godbold 1991). Because organomercurials are more lipophilic, they may have higher affinity for subcellular organelles, especially the membrane-rich mitochondria and chloroplasts, which are critical for energy production and metabolism.

Mercury Phytoremedation: Expression of a *merA* transgene allows plants to convert Hg(II) to volatile Hg(0)

The transfer of the bacterial organomercurial lyase (*merB*) and mercuric reductase (*merA*) genes into plants may enable these organisms to volatilize or sequester mercury,

providing a mechanism for removing concentrated mercury deposits from the soil (U.S. Pat. #'s: 5, 668, 292 and 5,965, 796). This experimental approach holds great promise for reducing mercury-related hazards and stands as a model for the future application of transgenic plants to beneficial environmental goals. Early studies showed that the bacterial merA gene confers $HgCl_2$ resistance to plants by enabling them to eliminate mercury as volatile Hg(0).

In order to express mercuric reductase (*merA*) efficiently in plants, a bacterial *merA* DNA sequence was modified to reduce the GC content in 9% of the coding region and to add plant regulatory elements (Rugh *et al*. 1996). When transferred to the laboratory model plant, *Arabidopsis thaliana* L., and to tobacco (*Nicotiana tabacum* L.), the new gene construct (*merA9*) conferred resistance to 50 µM Hg(II) (tobacco data unpublished), suggesting that *merA* plants enzymatically reduce Hg(II) and evaporate away Hg(0). Rugh et al. also examined the ability of yellow-poplar (*Liriodendron tulipifera* L.) tissue cultures and plantlets to express modified *merA* gene constructs (Rugh *et al*. 1998b). Three reconstructed genes were used for the transformation of yellow-poplar proembryogenic masses (PEMs), each having different amounts (0%, 9%, and 18% blocks, respectively) of altered coding sequence. Each of these constructs was shown to confer Hg(II)-resistance to transformed tissue cultures. Regenerated yellow-poplar plantlets containing the *merA18* gene germinated and grew vigorously in media containing 50 µM Hg(II). Given the distant evolutionary relationship of yellow-poplar, tobacco, and *Arabidopsis thaliana*, most, if not all, plants should be capable of *merA* transgene expression.

Closed-chamber assays were performed to compare the rates at which *merA* and wild-type plants reduce Hg(II) and evaporate Hg(0) from spiked media. *Arabidopsis* seedlings were transferred to reaction tubes containing 25 μM $HgCl_2$ in buffered medium (Rugh *et al*. 1996). The medium and headspace were evacuated each minute over a ten minute period and sampled for Hg(0) using a portable mercury vapor analyzer. *MerA9* plants volatilized 3-4 times as much Hg(0) as wild-type plants. In an analogous experiment, *merA18* yellow-poplar plantlets were placed in test tubes containing gel medium spiked with 10 μM Hg(II). The tubes were evacuated every 12 hours over a period of 6 days (Rugh *et al*. 1998b). Hg(0) readings indicated that *merA18*-plantlets released elemental mercury at approximately 10 times the rate of untransformed control plantlets. These experiments independently verified that a functional mercuric reductase is expressed in transgenic plant lines of both *Arabidopsis thaliana* and yellow poplar.

Hydroponic assays were used to quantify the rates at which *merA* tobacco remove Hg(II) from spiked medium (Heaton *et al*. 1998; Rugh *et al*. 1998a). Approximately 10 cm tall plants, from independent *merA9* and wild-type lines, were removed from soil and placed in an aqueous nutrient medium for a one week acclimation period. On day 8, the old hydroponic solution was replaced with fresh medium containing 5 μM Hg(II). Both the medium and plants were sampled or harvested at several time points over ten days. In each individual treatment, the entire dose of Hg(II) was bound to or absorbed by the plant's root system in fewer than 18 hours. Wild-type plants wilted on the first day, then recovered during the following 2 days and slowly transpired Hg(0), volatilizing ~20-25% of their initial mercury doses by the end of the experiment. By comparison, all of the *merA9* tobacco plants reduced between 30% and 50% of their initial Hg(II) load after one

day. Over the course of the assay, they never exhibited symptoms of stress and showed declining mercury tissue concentrations at each sample point, evaporating ~70-80% of the Hg(II) dosage from the hydroponic system by day 10. Though natural environments are much more complex than a hydroponics system, we expect that *merA*-plants will grow robustly, detoxifying and evaporating mercury from contaminated soils. Should our expectations hold true, the establishment of *merA* communities of plants will help prevent the accumulation of mercurial compounds in soils and sediments without having to be tended or harvested.

Mercury Phytoremediation: Volatilization vs. Sequestration

Presumably, *MerA* plants will elevate rates of environmental Hg(0) volatilization well beyond background levels. In certain situations this may not be a desirable result. Some areas may have a "funneling" topography or unique meteorological conditions that promote the rapid redeposition of Hg(0), making the goal of atmospheric dilution unachievable. These locations may require an alternative strategy, in particular, the deployment of plants that retain and tolerate high mercury loads in harvestable tissues. The ability of wild plants to accumulate mercury is largely dependent upon their natural physiochemical and structural characteristics (Du and Fang 1982; Du and Feng 1983). Species that have been examined have not been found to hyperaccumulate mercury to the extent that some do for other heavy metals, such as copper, nickel, and zinc. Plants, in general, may require special genes to enhance their uptake, retention, and tolerance of Hg(II). Specifically, plants will have to be engineered to load Hg(II) into tissues (*e.g.* cell wall) or organelles (*e.g.* vacuole) in the shoot where it can be safely sequestered.

Chelators, such as metallothioneins, phytochelatins, and organic acids may be helpful for transporting Hg(II) to these stuctures and for preventing Hg(II) from reacting with critical proteins. The overproduction of these small molecules in concert with the expression of the bacterial mercury-transport genes, *merP* and *merT*, may make plants efficient accumulators of mercury.

While the major benefit of phytosequestration is the minimal release of mercury, potential problems do exist. For one, herbivores may consume Hg(II) bound in leaves and shoots. Methods to avoid this predicament might include the frequent harvesting of tissues (to keep tissue concentrations low) or the sowing of edible, non-accumulating crops at the periphery of sites (to attract herbivores away from hyperaccumulators). A second disadvantage of phytosequestration is the added cost of managing, retrieving, and disposing of Hg-containing tissues. These expenses increase proportionally with the inaccessability of a site, though are still likely to be far less than those associated with engineering-based approaches.

Mercury Phytoremediation: Crop Design and Selection

In our inital efforts to develop mercury-detoxifying plants, we used *Arabidopsis thaliana*, tobacco and yellow-poplar principally because they are amenable to genetic transformation and plant regeneration. These species are not necessarily adapted to the conditions at most contaminated field sites. In areas for which phytovolatilization is most suitable, the "harvestability" of the plant will not be an issue, and site-compatibility will be the primary criterion for selecting plants. Mercury pollution is frequently deposited in lowland regions, including riparian and estuarine sites, salt marshes, and freshwater

wetlands. Facultative and obligate wetland grasses such as cattail (*Typha* spp.), rush (*Juncus* spp.), bulrush (*Scirpus* spp.), and cordgrass (*Spartina* spp.) have been used in constructed wetlands for sewage and wastewater treatment. Of these species, *Spartina* is best adapted to polluted saline and brackish ecosystems. Emergent aquatic plants help to stabilize sediments, contribute organic matter, and aerate sediments making conditions less conducive to mercury methylation (Reddy *et al*. 1990; Stengel 1993). Aquatic macrophytes possess many physiological attributes that should complement novel detoxifying abilities conferred by transgenes. Many species that are well-known to ecotoxicologists have never been used for bioengineering and new protocols will have to be developed for their genetic transformation and tissue culture regeneration.

Trees also possess physiological and structural characteristics that make them good candidates for phytoremediation (Stomp *et al*. 1993). For one, their abundance of non-living biomass enables them to load heavy metals without experiencing toxic shock. As an illustration of this attribute, they were shown to be effective for extracting heavy metals from sewage, in which they were grown, cut, and re-sprouted in continuous cycle (Riddell-Black 1994). Secondly, their long lives allow for prolonged service without replanting, a characteristic that carries added significance when considering strategies to contain genetically engineered plants. Since trees have long periods of non-reproductive juvenility, they may be harvested prior to the onset of flowering and seed production. Furthermore, some species, such as willow (*Salix* spp.) and poplar (*Populus* spp.), are particularly water-tolerant and may be valuable for treating inundated bottom lands. These species could be used in combination with grasses to construct a dynamic community for the phytoremediation of freshwater wetlands.

Concluding Remarks

Since the Minamata Bay disaster, industrialized nations have looked for alternatives to mercury and have sharply curtailed its use. Some developing countries continue to use mercury in a number of processes without controlling its release into the environment. This practice may eventually affect indigent, local communities that depend upon unmonitored waterways and floodplains for food and water supplies. The inhabitants of these communities are at a particular risk of methylmercury exposure if they consume fish from contaminated waters. Most polluted areas are not eliglible for high-cost, intensive engineering-based cleanup strategies, such as those that were employed at Minamata Bay. Less disruptive and more affordable alternatives must be devised. The use of biological organisms may provide a means to greatly reduce costs, minimize incidental mobilization, and help stabilize sites for ecological recovery as they are cleaned up. While bacteria appear promising for the treatment of industrial wastes, their specialized growth requirements may make them impractical for many environmental applications. Furthermore, the recovery of microbe-bound toxins, such as heavy metals, will be challenging and may not even be feasible in the field.

Plants possess a natural physiochemical apparatus that is well adapted to infiltrate and purify contaminated land and water. Plants are easier to contain than environmentally released microorganisms and centuries of agricultural practices have made their harvest a routine and efficient practice. Many studies have demonstrated that plants have great potential for extracting and detoxifying pollutants. However, there are no known plant species that naturally degrade or hyperaccumulate mercury and mercurial compounds. Bacterial pathways for detoxifying mercury and other poisons are

genetically and biochemically well characterized. The tools of molecular genetic engineering have enabled us to combine microbial capabilities with the physiological and structural advantages offered by plants. We have demonstrated that bacterial mercury-resistance genes can be used to confer broad-range mercury detoxifying and reducing capabilities to a range of plant species. This achievement provides the first evidence that plants can be engineered with novel detoxifying abilities for the purpose of remediating environmental pollution. One hindrance to the use of *mer* plants is that Hg-polluted sites often contain toxins besides mercury, many of which may be lethal to plants. As the field of environmental biotechnology develops, however, it is likely that researchers will discover or engineer genes to provide resistance to other compounds. By adding an appropriate set of detoxification genes, we may enable plants to tolerate and/or degrade several toxins present at a given site.

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CHAPTER 2

PHYTOREMEDIATION OF METHYLMERCURY POLLUTION: *MERB* EXPRESSION IN *ARABIDOPSIS THALIANA* CONFERS RESISTANCE TO ORGANOMERCURIALS [1]

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ABSTRACT

Methylmercury is an environmental toxicant that biomagnifies and causes severe neurological degeneration in animals. It is produced by bacteria in soils and sediments that have been contaminated with mercury. In order to explore the potential of plants to extract and detoxify this chemical, we engineered a model plant, *Arabidopsis thaliana,* to express a modified bacterial gene, *merBpe*, encoding organomercurial lyase (MerB) under control of a plant promoter. MerB catalyzes the protonolysis of the carbon-mercury bond, removing the organic ligand and releasing Hg(II), a less mobile mercury species. Transgenic plants expressing *merBpe* grew vigorously on a wide range of concentrations of monomethylmercuric chloride and phenylmercuric acetate. Plants lacking the *merBpe* gene were severely inhibited or died at the same organomercurial concentrations. Six independently isolated transgenic lines produced *merBpe* mRNA and MerB protein at levels which varied over a 10-15 fold range, and even the lowest levels of *merBpe* expression conferred resistance to organomercurials. Our work suggests that native macrophytes (e.g. trees, shrubs, grasses) engineered to express *merBpe* may be used to degrade methylmercury at polluted sites and sequester Hg(II) for later removal.

INTRODUCTION

Mercury is among the most hazardous of the heavy metals (1), primarily because its charged species have great affinity for the thiol group on cysteine residues of proteins and other important biological molecules (2, 3). Early studies demonstrated that mercury species inactivate metabolic enzymes and structural proteins (4, 5). The strong interaction of mercury species with cellular ligands may also account for its tendency to accumulate in organisms. Organomercurials are one to two orders of magnitude more toxic in some eukaryotes and more likely to biomagnify across trophic levels than ionic mercury (Hg(II) (1, 6, 7). The biophysical behavior of organic mercury is thought to be due to its hydrophobicity and efficient membrane-permeability.

Industrial and agricultural activities have released several hundred thousand tons of mercury into the biosphere during the past century (8). The most notable ecological disasters have been caused by the efflux of mercury-contaminated wastes into semi-contained fresh and saltwater basins. Between 1956 and 1968, widespread poisoning at Minamata Bay, Japan brought international attention to the hazards of mercury pollution. During this period, medical researchers discovered that high levels of monomethylmercury (CH_3-Hg^+) in seafood were responsible for severe neurological degeneration in birds, cats, and humans (9). This mercury species, traced from Minamata Bay sediment to wastewater from a chemical manufacturing plant, is the principal form of mercury that accumulates in fish and biomagnifies in their predators (10, 11).

Methylmercury has also been detected in lakes and estuaries into which only inorganic forms of mercury have been released. Microbes present in the sediment were found capable of processing Hg(II) to CH_3-Hg^+ (12, 13). Sulfate-reducing bacteria

isolated from the aerobic/anaerobic interface of these sediments were later found to be the principal methylators (14, 15). Using *Desulfovibrio desulfuricans* LS as a model system, methylcobalamin was identified as the intermediate in transferal of a methyl group from CH_3-tetrahydrofolate to Hg(II) (16, 17). While sulfate-reducing bacteria manage to survive in the presence of methylmercury by converting it to less soluble products (18), they do not carry out these reactions efficiently enough to prevent harmful levels of methylmercury from leaching into the surrounding environment.

Mercury resistant bacteria eliminate organomercurials by producing an enzyme, organomercurial lyase (MerB), that catalyzes the protonolysis of the carbon-mercury bond (19). The products of this reaction are a less neurotoxic inorganic species, Hg(II), and a reduced carbon compound.

MerB

$$\mathbf{R\text{-}CH_2\text{-}Hg^+ + H^+ \dashrightarrow R\text{-}CH_3 + Hg(II)}$$

These bacteria also synthesize a second enzyme, mercuric ion reductase (MerA), that catalyzes the reduction of the inorganic product, Hg(II), to a volatile and much less reactive elemental form, Hg(0) (20). The enzymes are encoded by the *merB* and *merA* genes of the plasmid-borne, broad-spectrum mercury-resistance (*mer*) operon (21). Both enzymes require a low molecular weight thiol-organic cofactor (e.g., cysteine, β-mercaptoethanol) for *in vitro* activity and may require sulfhydryl-bound substrates (19, 20).

Although many governments now require companies to recover mercury from their sludge and liquid wastes, they have been less exacting regarding the cleanup of previously polluted landfills and waterways. This lack of resolve is due partly to the fact

that the physical and chemical remediation techniques currently used to extract or immobilize mercury are extremely expensive, environmentally disruptive, and sometimes ineffective. A phytoremediation system in which plants extract, sequester, and/or detoxify mercury pollutants (22) may be a more attractive solution. Besides being cost effective, phytoremediation offers a promising alternative because plants naturally dominate most ecosystems, use solar energy, have large reservoirs of reducing power from photosystem I, have extensive root systems capable of extracting a variety of metal ions, and can stabilize and rehabilitate damaged environments (23, 24). In a previous paper (7), we communicated that the expression of a modified bacterial *merA9pe* gene enables *Arabidopsis thaliana* plants to grow on toxic levels of Hg(II) by converting this species to elemental mercury. We now report that plants transformed with a variant of the bacterial *merB* gene express organomercurial lyase and grow on concentrations of phenylmercuric acetate or methylmercuric chloride that are lethal to their wild-type progenitors.

MATERIALS AND METHODS

Strains and Plasmids: The *E. coli* strain SK1592 *(F-, gal-, thi-, sup-, tonA-, hsdR4, endA-, SbcB15)* was provided by Sidney Kushner as a spontaneous T1 phage-resistant derivative of SK1590 (25). The plasmid pDU202, which was used in metal ion sensitivity disk assays, contains a narrow-spectrum *mer* operon (26). This operon contains the *merA*, *merD*, *merP*, *merR*, and *merT* genes, but not *merB*. Accordingly, it confers resistance to Hg(II) but not to organomercurials (21). The pBluescriptSKII(-) (pBSSKII) vector was obtained from Stratagene, Inc. The binary vector pVST I,

designed for *Agrobacterium*-mediated plant transformations, was constructed by Malik and Wahab (27).

Reconstruction of *merB* for plant expression: Mercury-resistance plasmids have been isolated from a variety of bacterial species and strains. One well characterized *merB* gene (GenBank accession #U77087) is found on the broad-spectrum resistance plasmid R831b (28). This gene was subcloned on a 1.5 kb EcoRI fragment into pBR322 to make pCT12 (29). The 214 codon *merB* gene was amplified by the polymerase chain reaction (PCR) using long synthetic primers which modified the *merB* flanking sequences as shown in Figure 1. The sense primer, merB5'S, consisted of the 57 nt sequence 5'GCGGTCGGAT CCGAATTCGT CGACTAAGGA GGAGCCACAA TGAAGCTCGC CCCATAT3' and contained *Bam*HI , *Eco*RI, and *Bgl*II cloning sites, a TAA stop codon to end the translation of an upstream β-galactosidase fusion protein in *E. coli*, a GGAGGA bacterial translation signal to assist expression in *E. coli,* an AGCCACA consensus sequence for plant translation (30), an ATG start codon, and the first 18 nt of the *merB* coding sequence to prime the forward PCR reaction (7). The antisense primer, merB3'N, had the 43 nt sequence 5'CGTATCGGAT CCGAATTCAA GCTTATCACG GTGTCCTAGA TGA3', with *Hind*III, *Eco*RI, and *Bam*HI cloning sites and anticodons to the last 7 *merB* codons to prime the reverse PCR reaction. PCR was carried out for 35 cycles with denaturing, annealing, and extending temperatures/times of 95°C 1 min, 42°C 1 min, and 72°C 1 min. The amplified fragment, *merBpe*, was cleaved in the flanking *Bam* HI and *Hind*III sites and ligated into the multilinker of pBSSKII to make pBS*merBpe.* The pBS*merBpe* construct was electroporated into a strain of SK1592 that had previously been transformed with pDU202. The same *E. coli* strain was also

transformed with an empty pBluescriptSKII(-) plasmid to serve as a control in metal ion-sensitivity filter disk assays (Table 1). The *merBpe* sequence was transferred from pBS*merBpe* to the plant vector pVSTI (27) using the 5' *Bam*HI site and the 3' *Xho* I site in the Bluescript multilinker, to create pVSTI*merBpe*.

Disk Assays: The acetate salt of phenyl mercury (PMA; Sigma Chemical Co.), the chloride salt of methylmercury (CH_3HgCl; Alpha Aesar), and sodium p-chloromercury benzoate (NaPCMB); Sigma Chemical Co.) were prepared in DMSO or ethanol. Due to the extreme toxicity and membrane permeability of these chemicals, dry stocks and stock solutions were handled using protective clothing, eye protection, and 4H or Viton gloves (Fisher Scientific). All metal ion-sensitivity filter disk assays were performed in the presence of ampicillin to maintain the pBSSKII plasmid and streptomycin to retain pDU202, as described in Rugh et al. (1996). The data reported are the average results of several replicates, which, in all cases, varied by less than 1 mm.

Construction of Transgenic Plants: pVSTI*merBpe* was electroporated into an LBA4404 *Agrobacterium tumefaciens* strain (GIBCO BRL). Transformants were verified by Southern blotting and grown up in YEP medium (10 g Bacto-peptone, 10 g yeast extract, 5 g NaCl/ L) in the presence of streptomycin and kanamycin to maintain the T-DNA and pVSTI plasmids, respectively. Wild-type *Arabidopsis thaliana* (ecotype RLD) were transformed with this *A. tumefaciens* strain using the vacuum infiltration procedure (31).

Germination / Growth Experiments: Wild-type (RLD), *merA9pe* (transgenic control) (7), and *merBpe* (B4 line) Arabidopsis seeds were sterilized, vernalized at $4^{o}C$ for at least 24 h, and germinated on 1% Phytagar plates (GIBCO BRL) made with

Murashige and Skoog (4.3g/L, GIBCO BRL) medium containing PMA or CH_3HgCl. Seedlings were grown at 22^{o}C with a 16 h light / 8 h dark regime and photographed at 3 to 8 weeks.

Quantitative Northern Blot: Total RNA was prepared from transgenic and control plants (>15 seedlings/line) (32), resolved by electrophoresis on a 1% agarose-formaldehyde gel, and blotted to a Biotrans nylon membrane (ICN Biomedicals) (33). The membrane was probed with the 1.8 kb *Bam HI/Xho I merBpe* fragment. P^{32}-dATP was incorporated into the probe by a random primer method to yield a specific activity of approximately 5 x 10^8 cpm/ug (34).

Quantitative Western Blot / Isolation of MerB-specific Monoclonal Antibodies: Crude protein was prepared from *merBpe* and control plants (15 seedlings/line) in a buffer containing 5 mM EDTA, 10 mM $MgCl_2$, 10 mM NaCl, 25 mM Tris-HCl (pH 7.5) and 1 mM phenylmethylsulfonyl fluoride. Extracts were denatured by adding an equal amount of 2x SDS Sample Buffer and boiling for 5 min then separated on a 12% SDS-polyacrylamide gel (35). Resolved protein was electroblotted onto a Immobilon-P polyvinylidene fluoride membrane (Millipore Corp.).

Monoclonal antibodies were prepared against MerB following the procedure developed by Kohler and Milstein (36). Spleen cells were isolated from mice that had been immunized with a hexaHIS-tagged version of MerB prepared by Qiandong Zeng. The hybridoma cell line 2H8 was found to secrete an IgG antibody that strongly recognized the original HIS-tagged antigen, organomercurial lyase expressed from pBS*merBpe* in *E. coli,* and a protein expressed by *Arabidopsis* plants that were resistant to PMA. This antibody, Mab2H8, was purified over a Protein A column (Bio-Rad) and

used for quantitative western blots. Mab2H8 was reacted with membrane-bound plant protein for 90 min after blocking for 4 hr with 5% dry milk, 10% goat serum (Sigma) TBS-T buffer.

RESULTS

The *merBpe* construct used to transform *Arabidopsis thaliana* was modified from the original bacterial *merB* sequence by adding flanking regions containing consensus plant and bacterial translation signals as well as convenient restriction sites to facilitate cloning (Fig. 1). Sequencing identified a single silent transversion at bp 369 in the 642 bp coding region which probably occurred during the PCR amplification. In order to demonstrate that this clone made an active MerB enzyme, a metal ion-sensitivity filter-disk assay was used to compare the growth of *E. coli* strains with and without the *merBpe* construct in the presence of organomercurial compounds. A strain carrying a narrow-spectrum mercury-resistance plasmid, pDU202, which confers resistance to Hg(II) but not organomercurials, was used as the recipient for either pBS*merBpe* or an unmodified Bluescript plasmid. Bacteria expressing an active MerA enzyme from pDU202 and MerB enzyme from pBSmerBpe should eliminate organomercurials via a coupled reaction which produces Hg(0). As expected the SK1592/pDU202/pBS*merBpe* strain grew much closer to filter disks with PMA or CH_3HgCl than did the control strain (Table 1) indicating that the modified *merBpe* produces an active organomercurial lyase.

The *merBpe* sequence was subcloned into the plant transformation vector, pVST I, which provides a constitutive promoter (CaMV 35S), nopaline synthetase (NOS) 3' termination and polyadenylation signals, and a selectable kanamycin resistance marker

(NPT II). *Arabidopsis thaliana* plants were transformed by vacuum infiltration and the T_1 generation seeds they produced were screened for kanamycin resistance. Seven resistant germinants were selected for further study and used to establish independent *merBpe* lines. T_2 progeny of the B4 line were tested for organomercurial resistance by germinating on medium containing PMA or CH_3HgCl. As seen in Fig. 2, the B4 seedlings grew vigorously in the presence of 2 μM PMA (Fig. 2B and 2C), producing strong shoots, round, deep-green leaves, and fully-branched root systems. By contrast, RLD and *merA9pe* control seedlings flanking the B4 seedlings either failed to germinate or germinated, bleached white, and died within 2 to 3 weeks. *MerBpe Arabidopsis* had a clear advantage over the control plants at concentrations between 0.1 μM and 5 μM PMA or CH_3HgCl (0.5 and 2 μM are shown in Figure 2), flowering and setting seed after 7-8 weeks. However, at 5 μM CH_3HgCl (not shown), the *merBpe* seedlings and plants were clearly stressed, forming spindly shoots, lanceolate leaves, and relatively few lateral roots.

Independent transformants may vary with respect to steady-state RNA and protein levels due to differences in the genomic positioning and copy number of the transgene. Such variation commonly results from *Agrobacterium tumefaciens*-mediated transformation (37). Differences in expression levels were confirmed by Northern and Western blots shown in Fig. 3. Total RNA samples from 5 *merBpe* lines and the RLD parent line were blotted and hybridized with a P^{32}-labeled *merBpe* probe. A strong 700 bp mRNA band was detected in samples from the *merBpe* lines but not in those from RLD (Fig. 3A). This is the size predicted from the sequence of the *merBpe* construct.

Based on the normalized intensities of these bands the levels of *merBpe* mRNA varied over an 11-fold range (Figure 3C).

MerB protein was assayed in crude extracts from 6 *merBpe* lines and RLD. Western blots were probed with the Mab 2H8 MerB-specific monoclonal antibody that was prepared for this study (see Materials and Methods). The 22,400 kDa organomercurial lyase was detected in all transgenic lines (Fig. 3B). MerB protein levels varied over a 14-fold range and correlated linearly with levels of *merBpe* mRNA (r^2 = 0.955; Fig 3C). Independent repetitions of the Western blot confirmed the rank order of expression levels for the various lines shown in Fig. 3B. The strong correlation between mRNA and protein levels suggests that the transgene is not subject to unusual variations in post-transcriptional regulation.

Given that *merBpe* confers resistance to organomercurials, we hypothesized that lines expressing more organomercurial lyase should grow faster on various levels of organic mercury (see Discussion). We looked for a relationship between growth rate and expression levels by germinating several *merBpe* lines on 1 μM PMA (Fig. 2H and I). However, there were no observable growth differences among the *merBpe* lines that correlated with expression levels. Similar results were obtained at PMA concentrations between 0.1 and 1 μM. Plants from the B1, B4, and B5 lines all appeared healthy, although they were slightly inhibited compared to unchallenged plants. One other line, B3 (not shown) gave similar results. Plants from the B8 line with intermediate levels of gene expression grew poorly on PMA, yielding small, slow-growing seedlings with spindly, light green leaves. All lines tested, including a wild-type control, thrived on mercury-free agar plates, sprouting strong deep green shoots and leaves.

DISCUSSION

A bacterial organomercurial lyase gene, *merBpe,* reconstructed with an upstream sequence typical of plant genes, enables transgenic plants to germinate and grow well on 0.1-2 μM CH_3HgCl, concentrations that retard or kill control plants. A simple interpretation of this result is that *merBpe* plants efficiently protonolyze organic mercury, producing a more tolerable mercury species, Hg(II). Consistent with this interpretation, we found that 0.5 μM CH_3HgCl or PMA was required to kill *Arabidopsis* seeds and seedlings as efficiently as 50 μM $HgCl_2$ (Rugh et al., 1996). The greater toxicity of organic mercury relative to Hg(II) to eukaryotes is usually attributed to its hydrophobicity and to its tendency to coordinate with a counter-ion to form a neutral species (38). These characteristics enable organic mercury to pass through cell membranes and reach higher intracellular concentrations than externally applied Hg(II). Furthermore, eukaryotes possess membrane-rich organelles that may form localized sinks for organomecurial deposition (23).

Considering that Hg(II) is generated by MerB within the cytoplasm in the presence of vulnerable cellular proteins, it remains paradoxical that *merB* plants tolerate more organic mercury than wild-type plants. We suspect that in the intracellular environment, the lipid solubility of organic mercury gives it access to membrane-bound eukaryotic organelles such as mitochondria and chloroplasts, where it may poison essential oxidative and photosynthetic electron transport chains more easily than Hg(II) (7, 23). It is also plausible that cytoplasmic chelators, including phytochelatins and metallothioneins, bind and sequester Hg(II) in preference to the organomercurials. MerB expression would confer an advantage by making mercury available to these protective

compounds. A challenging problem to future research on this phytoremediation system would be to quantify the cellular levels of CH_3Hg^+ and Hg(II), and identify the various compounds to which they are bound.

The Michaelis constants describing the reaction velocities of MerB acting on a variety of organomercurial substrates, including methylmercury and PMA, are very high (K_m = 0.5-3.3 mM) (19), suggesting a relatively weak affinity for substrate. However, organomercurial resistance operates in environments and under experimental conditions with low substrate concentrations where only a small fraction of the enzyme may be bound to organic mercury. Our motivation for generating and maintaining a variety of *merBpe* lines was to determine whether, at these low substrate concentrations, organomercurial resistance depends upon the amount of MerB present in the cytoplasm. Although we detected up to 14-fold differences in MerB expression, we did not observe the direct relationship between enzyme levels and faster growth on medium containing organic mercury that would have been predicted by a simple steady-state model. Specifically, the lines with the most MerB protein were not more resistant to various organic mercury concentrations than those with lower but measurable MerB levels. Apparently, the abundance of MerB enzyme is not the limiting factor in organomercurial degradation. A reasonable explanation for this incongruity is that the reaction is limited by one of several kinetic parameters and is not functioning at steady-state (39). For example, in a living cell, the kinetics of the MerB catalyzed reaction could be constrained by the rate of diffusion of organomercurial substrates from cellular membrane systems to sites of catalysis or by the rate of diffusion of the product, Hg(II), away from the enzyme.

The remediation of mercury-polluted sites has been slow because the chemical-engineering technologies currently used to remove mercurials are expensive and disruptive. Excavation and roasting of soil is considered the "best demonstrated treatment technology" (40), but it is impractical for very large sites. Vitrification and concrete capping, which aim to stabilize mercury, render the site uninhabitable for plants, insects, and other organisms. An ideal treatment would degrade methylmercury, sequester other mercury forms, and help restore biological productivity. Our proposed strategy is to introduce engineered native plants that readily assimilate methylmercury and, by virtue of the *merBpe* gene, convert it into Hg(II). The plants would act as a biological filter, absorbing methylmercury from the sediment and water, and MerB would catalyze the protonolysis of the methylmercury as it enters plant cells. Hg(II) is 50 times less toxic to plants (this study and Rugh *et al*. 1996) and lower animals (38) and much less prone to biomagnification than methylmercury (1). Once formed, Hg(II) should accumulate in plant tissues. Plants could be harvested before Hg(II) reached toxic levels, and the mercury could be extracted or disposed of in a greatly reduced volume (41).

The phytoremediation system we have proposed requires the eventual removal of plant material that has accumulated high levels of inorganic mercury. Some benefits to this approach are the ability to immobilize mercury without disrupting the environment and the opportunity to recycle mercury by extracting it from the harvested plants. The size and topography of many contaminated sites, however, may make harvesting economically unfeasible. We have previously reported on *merA9pe* transgenic plants that electrochemically reduce Hg(II) to Hg(0), a volatile form that escapes from plant tissues (7). Judging by the phenotypes of independently transformed *merA9pe* and *merBpe*

plants, plants made to express both genes simultaneously will be capable of converting methylmercury into Hg(0), thereby volatilizing it from their local environment. These plants would require very little maintenance and could theoretically be engineered to volatilize mercury at a rate that is safe and consistent with governmental regulations. We and our collaborators are currently working to develop transgenic aquatic monocots for the phytoremediation of mercury-contaminated wetlands.

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Strain *merA* plasmid test plasmid phenotype	SK1592 pDU202-*merA*$^+$, pBSSKII-*merB*$^-$ Hg(II)^r, MeHg^s	SK1592 pDU202-*merA*$^+$ pmerBpe-*merB*$^+$ Hg(II)^r, MeHg^r
Metal Salt 2ul/disk	zone diameter	zone diameter
$HgCl_2$ 0.1 M	18 mm	20 mm
PMA 0.1 M	22 mm	8 mm
MeHgCl 0.1 M	26 mm	10 mm
NaPCMB .01 M	13 mm	10 mm

Table 1. Filter disk assay for mercury sensitivity in *E. coli*. *E. coli* strain SK1592 containing the various *mer* gene and control plasmids were assayed for mercury and organomercurial resistance. Each 6 mm filter diameter disk contained 2 µl of freshly prepared metal salt stock solution at the concentration indicated. The genotype of each strain and sensitivity (s) or resistance (r) phenotype on different forms of mercury is indicated below the strain and plasmid designations.

Figure 1. PCR primers and strategy for modification of the *merB* gene.

Two PCR primers were used to add synthetic flanking sequences to the *merB* gene that have elements necessary for bacterial and plant expression. The sense primer, MerB5'S, contained restriction endonuclease cloning sites, a TAA stop codon, bacterial (SD) and plant (PT) translation signals, an ATG start codon, and the first 18 nt of the *merB* coding sequence to prime the forward PCR reaction. The antisense primer, merB3'N, contained cloning sites, and 21 nt of anticodon sequence (covering the last 7 *merB* codons) used to prime the reverse PCR reaction.

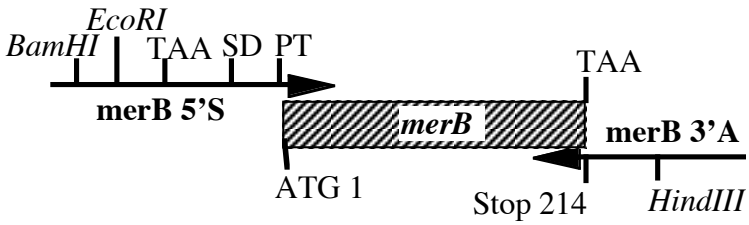
EcoRI
BamHI
TAA SD PT
TAA
merB 5'S
merB
merB 3'A
ATG 1
Stop 214
HindIII

Figure 1.

Figure 2. Growth of parental and transgenic *Arabidopsis thaliana* on organomercurials.

A-C. Growth on PMA: Seeds from the *merBpe* line B4 (center of each plate) germinated and grew on growth media containing (A) no mercury and (B, C) 2 μM PMA. Transgenic *merA9pe* seeds (left) and wild-type RLD (right) seeds used as controls, did not germinate on PMA. Plates in (A) and (B) were photographed at 4 weeks. The plate shown in (C) is the same as in (B), photographed 2 weeks later.

D-G. Growth on methylmercury: Seeds from the B4 line (center) also grew on substrates with (D) no mercury, (E) 0.5 μM CH_3HgCl, and (F, G) 2 μM CH_3HgCl. Plates were photographed after 5 (D-F) and 8 (G) weeks incubation. Transgenic *merA9pe* (left) and RLD (right) seeds do not grow on these concentrations of CH_3HgCl.

H-I. Growth comparisons among independent lines on PMA: Seeds from 4 independently isolated transgenic lines (B1, B4, B5, B8) representing a range of *merBpe* expression levels germinated and plants grew on (H) no mercury and (I) 1 μM PMA, while the wild-type RLD seeds and plants grew only on the control plate (H). Plates in (H) and (I) were photographed at 3 weeks post-germination.

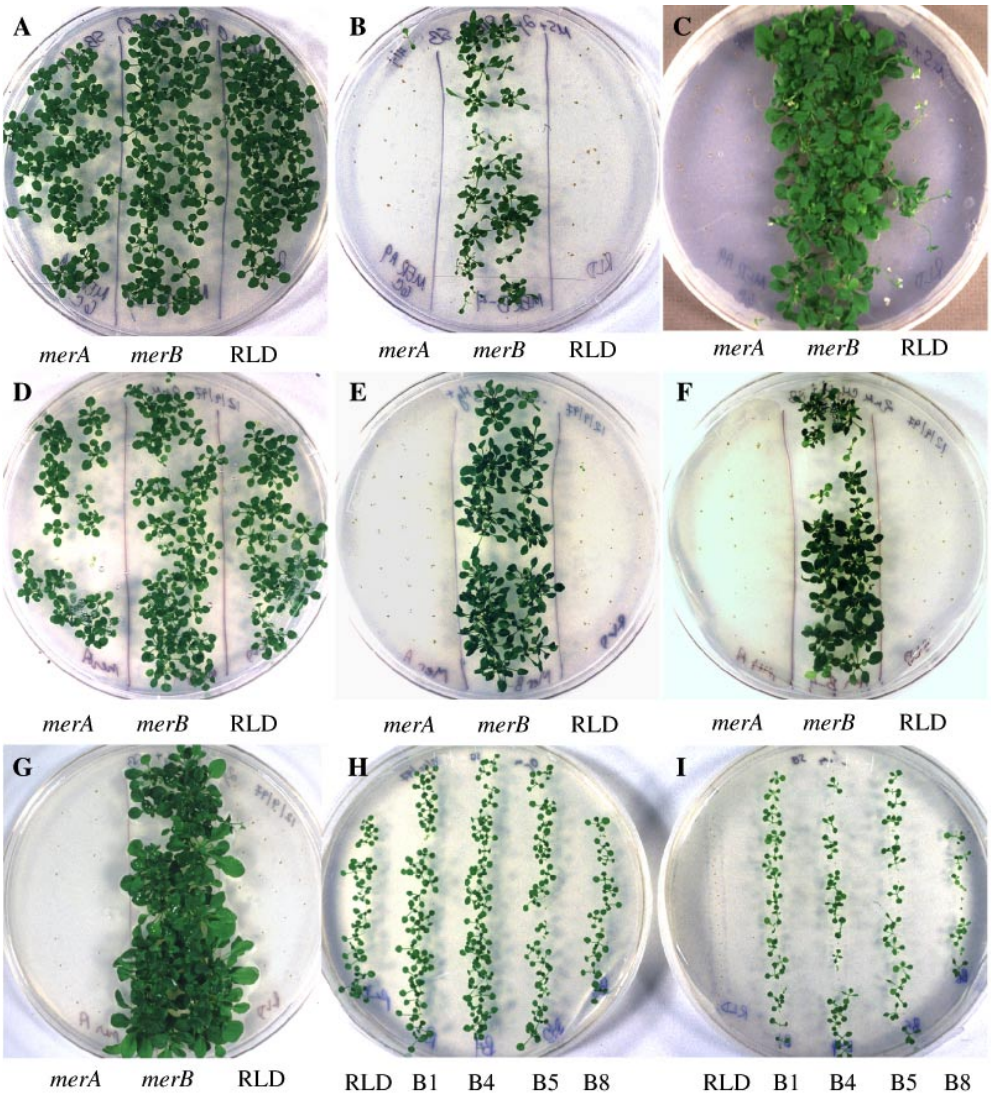
A
merA merB RLD
B
merA merB RLD
C
merA merB RLD
D
merA merB RLD
E
merA merB RLD
F
merA merB RLD
G
merA merB RLD
H
RLD B1 B4 B5 B8
I
RLD B1 B4 B5 B8

Figure 2.

Figure 3. Relative *merBpe* mRNA and MerB protein levels in independent transgenic plant lines.

A. *merBpe* mRNA levels: Five plant lines were tested for relative expression of *merBpe* mRNA by northern blotting and probing with a P^{32}-labeled *Bam HI/Xho I merBpe* fragment. Approximately 10-15 μg total RNA prepared from >30 seedlings (2-3 cm plants) was loaded for each sample. *MerB* mRNA was visualized and the relative band intensities were quantified using a Molecular Dynamics PhosphorImager. In order to control for variation in loading and transfer, these values were normalized to levels of 18S rRNA (not shown), determined by re-probing the filter with a oligonucleotide encoding the soybean 18S rRNA gene (42). An 11-fold difference was detected between the highest expressing line, B1, and the lowest expressing line, B5.

B. MerB protein levels: Western blots were performed on total protein extracted from 6 *merBpe* plant lines using the MerB specific monoclonal antibody, Mab2H8 (Materials and Methods). Fifteen 3-4 cm seedlings were harvested for each sample. The protein-antibody complex was visualized on X-ray film using a horse radish peroxidase-based chemoluminescent detection system (Amersham Life Sciences). Relative amounts of MerB were measured by scanning the film with a Molecular Dynamics Densitometer, integrating the intensities of the bands over their respective areas, and normalizing to total protein. Relative amounts of total protein were determined by quantifying the band intensities of a parallel gel stained with Coomassie blue (not shown) on a densitometer. Values recorded for MerB are the averages of 3 experiments while those for total protein are the averages of 2. All data reported for quantitative experiments described here are measurements of band intensities and reflect relative differences in expression between

merBpe lines. The B1 sample was shown to have ~14 fold more steady-state organomercurial lyase than the lowest expressing line, B3.

C. Relative mRNA and protein levels: A linear regression demonstrates that steady-state levels of mRNA and protein are correlated, confirming differences in *merBpe* gene expression and indicating that gene regulation is straightforward. The relative *merBpe* mRNA and MerB protein levels in independent transgenic lines (see Materials and Methods) were normalized to the level in the lowest expressing line in each category (B5, mRNA; B3, protein). The relevance of MerB expression levels to organomercurial resistance is discussed in the text.

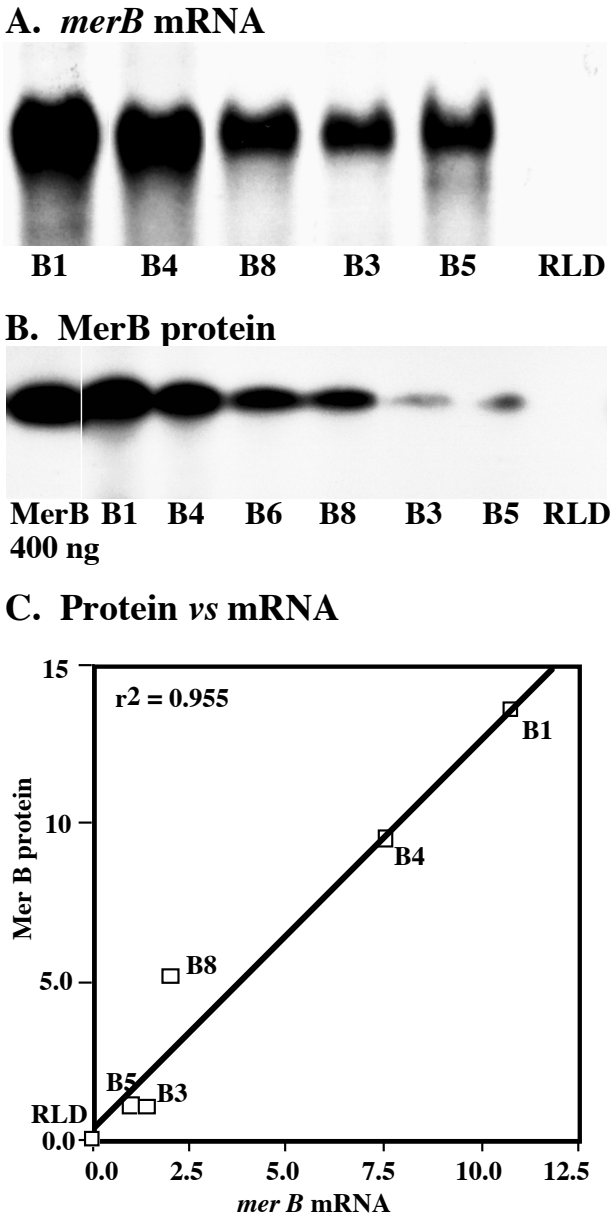
A. merB mRNA
B1
B4
B8
B3
B5
RLD
B. MerB protein
MerB 400 ng
B1
B4
B6
B8
B3
B5
RLD
C. Protein vs mRNA
r2 = 0.955
Mer B protein
mer B mRNA
15
10
5.0
0.0
0.0
2.5
5.0
7.5
10.0
12.5
B1
B4
B8
B5
B3
RLD

Figure 3.

CHAPTER 3

PHYTODETOXIFICATION OF HAZARDOUS ORGANOMERCURIALS BY GENETICALLY ENGINEERED PLANTS [1]

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[1]Scott P. Bizily, Clayton L. Rugh, and Richard B. Meagher. 2000. Nature Biotechnology, USA 18:213-217.

ABSTRACT

Methylmercury is a highly toxic, organic derivative found in mercury-polluted wetlands and coastal sediments worldwide. Though commonly present at pg/g (pptr) to ng/g (ppb) concentrations in the substrate, methylmercury can biomagnify six to seven orders of magnitude to levels that poison predatory animals and humans. In the interest of developing an in situ detoxification strategy, a model plant system was transformed with bacterial genes for an organic mercury detoxification pathway, mercuric reductase (*merA*) and organomercurial lyase (*merB*). *Arabidopsis thaliana* plants expressing both genes grow on 50-fold higher methylmercury concentrations than wild-type plants and up to 10-fold higher concentrations than plants engineered to express *merB* alone. An in vivo assay demonstrated that both bacterial transgenes are required for plants to detoxify organic mercury by converting it to volatile and less toxic elemental mercury. MerB levels showed a positive correlation with the rate of elemental mercury volatilization, but it is clear that other factors including substrate transport and diffusion may also be significant.

Introduction

During the past century the chemical, mining, and defense industries have utilized mercury as a catalyst, electrode, coolant, and amalgamator. Failing to recognize mercury's toxicity and potential environmental impact, most industrial facilities did not attempt to remove it from their waste streams. Factory effluents were commonly routed into nearby bodies of water, where mercury and other heavy metals were deposited in sediments. This practice was widely curtailed in the 1970s in response to two epidemics of human poisoning in Japan (Minamata and Niigata), which demonstrated that mercurials could biomagnify in aquatic and wetland ecosystems [1] as shown in Figure 1. Clinical investigations revealed that an organic derivative, methylmercury (CH_3Hg^+), was the principal form of mercury that accumulated in fish and their consumers, causing neurodegenerative symptoms [2-4]. Thirty years later, methylmercury contamination of aquatic sediments is now regarded as one of the most serious environmental problems facing both developed and emerging nations [5].

Methylmercury is a relatively rare waste product, but it can be generated in situ from a more commonly discharged toxicant, ionic mercury [Hg(II)]. Mercury methylation is primarily a biotic process mediated by anaerobic bacteria, such as the sulfate-reducers that are active in aquatic sediments [6-8]. Methylmercury and ionic mercury will both slowly leach into the water table despite their high affinities for organic components of sediment [9]. In soluble form, both toxicants are absorbed directly by plankton and through the gastrointestinal tracts of higher organisms, though uptake rates and retention times are much higher for CH_3Hg^+ [10-13]. As a result, CH_3Hg^+ is transferred far more efficiently than Hg(II) across trophic levels in the food chain. In

addition, toxicity studies in a variety of organisms suggest that CH_3Hg^+ poses serious health risks at 10 to 60-fold smaller dosages than Hg(II) [12,14,15]. Numerous sites in the U.S. that were contaminated with mercurials have not been remediated due to the high costs of dredging, transporting, and landfilling hazardous sediments. One relatively inexpensive and ecologically responsible clean-up alternative is phytoremediation, a technique in which selected plant species are introduced to extract and detoxify pollutants [16-19]. Although no naturally-occurring plant species are reported to detoxify CH_3Hg^+, the availability of cloned bacterial mercury resistance genes has presented the possibility of genetically engineering plants for methylmercury phytoremediation.

Bacteria isolated from organic mercury-contaminated environments possess two enzymes that convert methylmercury, phenylmercury, and other organomercurials to elemental mercury [Hg(0)] [20]. Hg(0) is much less toxic than either Hg(II) or organic mercury and rapidly diffuses out of bacterial cells due to its volatility. The bacterial mercury-processing enzymes, organomercurial lyase (MerB) and mercuric reductase (MerA), catalyze the following reactions:

MerB

$$\mathbf{R\text{-}CH_2\text{-}Hg^+ + H^+ \dashrightarrow R\text{-}CH_3 + Hg(II)}$$

MerA

$$\mathbf{Hg(II) + NADPH + H^+ \dashrightarrow Hg(0) + NADP^+}$$

By introducing the *merA* and *merB* genes, we have attempted to create plants that demethylate CH_3Hg^+ and reduce Hg(II). In theory, plants with both genes should extract organomercurials from substrates and transpire Hg(0) into the atmosphere using the same enzymatic mechanism as bacteria (Fig. 1). Because the atmospheric residence time of

Hg(0) is approximately two years, it can be diluted to trace levels before redepositing into the terrestrial substrate [17]. Furthermore, the quantity of mercury released on a yearly basis from polluted sites can be regulated and will, in all likelihood, be small in comparison with the atmospheric mercury load, which is approximately 4 x 10^6 kg [21]. Based on this observation, we would not expect the accelerated release of mercury by engineered green plants to greatly impact the global mercury cycle.

Results

Construction of *merA/merB* lines and assessment of methylmercury resistance phenotype. The bacterial *merA* and *merB* genes were modified for efficient plant expression and transferred independently into *Arabidopsis thaliana* using *Agrobacterium*. Plant lines that varied widely in the levels of transgene expression were characterized [15,22]. Lines with moderate to high levels of expression were used in standard genetic crosses to combine independent *merA* and *merB* T-DNA insertion alleles in the same background. Screening for both proteins with monoclonal antibodies, several F_1 plant lines expressing both MerA and MerB were selected *(merA/merB-1, -2, -3, -4, -5, -6*). Having received one haploid chromosome set from each parent, these F_1 plants are necessarily heterozygous for one or more *merA* and *merB* T-DNA insertions. These lines were subsequently selfed to generate six segregating F_2 populations containing both homozygous and heterozygous plants. Transgenic plants from a single line, *merA/merB-1*, were initially analyzed for resistance by germinating seeds on four concentrations of methylmercury (0, 1, 5, and 10 μM) (Fig. 2 A-D). In the absence of mercury, *merA/merB-1* plants did not differ in appearance from wild-type plants, suggesting that

the insertion of foreign genes had not produced deleterious effects. When challenged with organic mercury, plants with *merB* alone grew vigorously only at moderate concentrations (1 μM) of CH_3HgCl. *MerA/merB-1* seeds and plants, by comparison, had a substantially higher level of resistance, germinating and sustaining growth at 5 μM and 10 μM CH_3HgCl. At 5 μM CH_3HgCl they appeared dark green and vigorous with healthy shoots and dense roots and grew at approximately the same rate as unchallenged controls. At 10 μM CH_3HgCl they grew more slowly but were not chlorotic. *MerB* seeds germinated on both higher concentrations, though they were strongly inhibited, chlorotic, and eventually died. Wild-type (RLD ecotype) and *merA* seeds failed to germinate at 1 μM CH_3HgCl and were also killed at 0.25 μM CH_3HgCl (not shown), the minimum concentration that produced 100% lethality.

Analysis of *merA/merB* plants for conversion of organic mercury to Hg(0). Individual *merA/merB* plants from all six populations were analyzed by an in vivo enzyme assay that detects Hg(0), the volatile product of the coupled MerA/MerB reaction. The assay was initiated by placing a four week-old plant in a side-arm test tube containing organic mercury. Gaseous elemental mercury was sampled from the side port of the reaction tube over a 10 minute incubation period and quantified by a mercury vapor analyzer. Control plants from the wild-type, *merA*, and *merB* lines failed to generate detectable levels of Hg(0) above background. By comparison, most plants from the *merA/merB* populations that were assayed produced elemental mercury at average rates ranging from 10 - 59 pg Hg(0) • mg^{-1} plant tissue (fresh wt.) • $min.^{-1}$ (Fig. 3), representing values at least 100 to 1000-fold higher than background levels. Mean volatilization rates for *merA/merB* plants grouped into three categories: High

(*merA/merB-1*, *merA/merB-2*) = 57 - 59 pg Hg(0) • mg $^{-1}$ plant tissue (fresh wt.) • min.$^{-1}$; Intermediate (*merA/merB-3*, *merA/merB-5*) = 30 - 39 pg Hg(0) • mg $^{-1}$ plant tissue (fresh wt.) • min.$^{-1}$; Low (*merA/merB-4*, *merA/merB-6*) = 10 pg Hg(0) • mg $^{-1}$ plant tissue (fresh wt.) • min.$^{-1}$.

Relationship between Hg(0) evolution rate and methylmercury resistance phenotype. To investigate whether organic mercury resistance relates to the rate of Hg(0) volatilization, plants from the six *merA/merB* populations were grown on medium containing 0 or 5 μM phenylmercuric acetate (PMA; see Experimental Protocol) (Fig. 2E, F). In the absence of PMA, all six *merA/merB* lines were healthy and grew at rates similar to wild-type plants. When challenged with 5 μM PMA, plants from two populations, *merA/merB-1* and *merA/merB-2*, clearly outperformed the others. These same lines also had the highest average Hg(0) evolution rates in the coupled enzyme assay (Fig. 3). The *merA/merB-3* and *merA/merB-5* lines had intermediate Hg(0) evolution rates and grew on 5 μM PMA although much more slowly than the aforementioned lines. The remaining lines, *merA/merB-4* and *merA/merB-6*, failed even to germinate. Accordingly, these lines had evolution rates approximately three to six-fold lower than the other lines. These results suggest that methylmercury resistance is directly related to the capacity for converting this compound to elemental mercury.

Relationship between enzyme expression levels and Hg(0) evolution rate. We investigated the relationship between Hg(0) evolution rates and steady-state concentrations of MerA and MerB. Seventy-eight three week-old plants randomly selected from the six *merA/merB* populations were sampled for mercury evolution as described earlier. Hg(0) evolution rates in this set of samples were generally higher than

in the previous set due to the use of younger plants. Forty-eight plants that evolved significant levels of Hg(0) above background and 14 that did not were subsequently sampled for PEP, MerA, and MerB. PEP is a constituitively expressed protein that was used as an internal control. Relative protein concentrations were determined by performing western blots and quantifying protein bands on a densitometer (Fig. 4A, B, C; see Experimental Protocol). Each of the 14 plants that were negative for Hg(0) evolution were found to lack MerA and/or MerB. The lack of expression in these plants most likely resulted from the loss of *mer* alleles due to segregation. These plants provided convenient negative controls.

Mer enzyme levels, corrected for total cellular protein and loading (see Experimental Protocol), were normalized and grouped into bins. For MerB levels, bins were constructed to reflect 10-fold increases in expression. Volatilization rates for plants within each bin were averaged as shown in the bar diagram in Figure 4D and used to calculate standard errors. Plants without measurable MerA and/or MerB protein were grouped separately to reflect the fact that both enzymes were required to convert PMA to Hg(0). MerB levels showed a positive relationship with the rate of Hg(0) production, with approximately 10-fold increases in expression corresponding to 1.5 to 2-fold increases in the volatilization rate. Analysis of the effect of MerA expression was performed similarly. However, MerA bins were constructed to reflect 2-fold and 5-fold increases in protein expression, given that MerA levels did not vary as widely among plants as did MerB levels. Mean volatilization rates had no obvious relationship to MerA expression levels.

Though the relationship between MerB and Hg(0) evolution was positive, it was not strictly proportional. By comparison, MerA expression was clearly needed to volatilize Hg(0), yet increases in MerA levels did not impact volatilization. In order to better characterize the influence of both enzymes on volatilization rates, we performed a multiple regression analysis using MerA, MerB, and an interaction term as the independent variables and the Hg(0) evolution rate as the dependent variable. Because the data for each parameter did not approach a normal distribution, we tested several transformations for the ability to normalize each distribution. The logarithmic transformation gave the most satisfactory results and enabled us to apply a standard multiple regression model formulated below. Results are summarized in Table I.

$$\mathbf{\log(pg\ Hg(0)\ min^{-1}\ mg^{-1}) = k_1 \log(MerB) + k_2 \log(MerA) + k_3 \log(MerB) \bullet \log(MerA) + k_4}$$

Based on the regression analysis MerB has a significant positive relationship to Hg(0) evolution rate, while MerA does not, further supporting our earlier conclusion. The interaction term, log(MerB) • log(MerA), had a significant negative effect, reflecting the fact that increases in the Hg(0) volatilization are reduced at the highest enzyme expression levels. This suggests that a plant may have a saturable capacity for transporting mercurials that is not overcome by increasing enzyme levels.

Discussion

The aim of this study was to determine whether a bacterial enzymatic pathway for organic mercury detoxification could be reconstructed in plants by transforming them with the *merA* and *merB* genes. We had previously shown that *merA* [22] and *merB* [15]

confer distinct resistance phenotypes to *Arabidopsis thaliana* when present as independent transgenes. Expression of *merA* confers Hg(II) resistance and enables plants to reduce Hg(II) to Hg(0). Expression of *merB* alone confers a moderate level of organic mercury resistance. This report demonstrates that the coupled expression of *merA* and *merB* in the same genetic background enhances organic mercury resistance by up to 10-fold and, more importantly, allows plants to convert toxic organic mercury substrates to relatively inert elemental Hg(0). This brings us one step closer to the large-scale decontamination of methylmercury-polluted aquatic sediments.

These results could not be taken for granted, because *merA/merB* plants have neither the same subcellular architecture nor the full complement of mercury resistance genes that bacteria possess. It is furthermore likely that the subcellular localization of MerA and MerB in plant cells does not precisely replicate the bacterial system. While the exact cell biology and biochemistry of organomercurial phytodetoxification are largely uncharacterized, the increased methylmercury resistance and production of elemental mercury clearly suggest that organic mercury diffuses into plant cells and reacts with MerB and that the ionic mercury produced is reduced by MerA.

A comparison of independent plants from different *merA/merB* populations for methylmercury resistance reveals that plants from some populations grow much faster than others on relatively high concentrations (1-2 ppm) of CH_3HgCl or PMA. These differences in growth correspond to quantitative variations in the rate of Hg(0) evolution (or, equivalently, organic mercury transformation), suggesting that it is advantageous for plants to eliminate mercury as rapidly as possible.

Another important issue, whether Hg(0) evolution rates depend upon the abundance of MerA and MerB in the cytoplasm, was addressed by sampling Hg(0) evolution rates and enzyme levels for individual plants. The most simple (single) enzyme kinetics model predicts that, in vitro, initial reaction rates should be proportional to enzyme concentrations and that one of the two enzymes should be limiting. However, natural systems are highly compartmentalized, interactive environments in which enzymes and their substrates are affected by membrane barriers and other reactive molecules, and simple kinetics models do not necessarily apply. We have previously shown, for example, that for independent transgenic plant lines expressing *merB* alone, resistance to organic mercury is not proportional to enzyme concentration [15]. In constrast, in plants expressing *merA* alone, *merA* expression levels correlated strongly with Hg(0) evolution rates [22]. This discrepancy provided incentive to look at the relationship between enzyme expression and Hg(0) evolution in this present study.

We have shown in this manuscript that Hg(0) evolution rates for individual plants correlate positively with MerB levels. We had anticipated that MerB would be rate-limiting to Hg(0) evolution given that it is the first enzyme in the organic mercury detoxification pathway and that it is a much less efficient enzyme than MerA with a 500 to 1000 times higher K_m and 50 to 1000 times lower turnover number, depending upon the organomercurial substrate [23,24]. Mercury evolution correlates with exponential increases as opposed to proportional increases in MerB (Fig. 4D). One possibility is that the mercury transport/transpiration system is saturable. Although MerA activity is clearly required to convert organic mercury to Hg(0), MerA expression levels do not show a relationship to mercury evolution over the range of values tested.

Regression analysis supported these results, detecting a statistically significant relationship between the log(MerB) parameter and log[Hg(0)/min/mg] and no effect of the log(MerA) parameter upon the volatilization rate. In the context of this model, the interaction parameter, log(MerB) • log(MerA), also explained a significant proportion of the variation in log[Hg(0)/min/mg], but had a negative effect, reflecting the failure of high enzyme levels to produce high volatilization rates. Given that the regression model explained 42% of the variation in evolution rates, other physiological factors such as uptake, cell compartmentalization, and/or diffusion may be at least as important as enzyme abundance or catalytic efficiency and their effects have yet to be studied.

The field of phytoremediation is receiving attention because it offers a cost-effective and environmentally progressive clean-up solution for polluted land, air, and water [17]. Plants have been proposed both as a treatment for previously contaminated sediments and as a buffering system to absorb run-off from hazardous waste containment facilities. They offer several advantages over the use of microorganisms for bioremediation, such as autotrophic growth and ease of manipulation and containment. Moreover, they are physiologically adapted to extract nutrient metal ions from the soil, producing dense root systems that infiltrate large volumes of soil and help to stabilize disturbed ecosystems. Our work demonstrates that phytoremediation can be expanded beyond the natural physiology of plants by using multi-gene chemical resistance pathways available from other organisms.

Experimental Protocol

Construction of transgenic plants. The RLD ecotype of *Arabidopsis thaliana* was used as the progenitor for creating separate *merA* and *merB* lines that were analyzed previously [15,22]. To generate lines with both transgenes, standard crosses were performed among two different *merA* parents (*merA9-1, merA9-6*) and two different *merB* parents (*merB-1, merB-4*). The *merA/merB* lines were formed from the following parents: *MerA/merB-1* (from *merA9-1* and *merB-4*), *merA/merB-2* (from *merA9-1* and *merB-1*), *merA/merB-3* (from *merA9-1* and *merB-1*), *merA/merB-4* (from *merA9-6* and *merB-1*), *merA/merB-5* (from *merA9-1* and *merB-1*), *merA/merB-6* (from *merA9-1* and *merB-1*. All *merA/merB* plants subsequently examined were from the F_2 generation. Because we examined MerA and MerB levels (see below) in individual plants segregating for these alleles, no attempt was made to determine which plants were homozygous or heterozygous for each insertion.

Organic mercury resistance assays. Seeds were sterilized, plated in petri dishes containing standard agarose growth medium [$^1/_2$ X MS salts (GIBCO BRL), 10% sucrose, 0.8% Phytagar (GIBCO BRL), pH 5.7], and placed in a 22^{o}C growth chamber. Petri dishes were positioned vertically to encourage the growth of roots and were photographed at 3 to 4 weeks. Methylmercuric chloride and phenylmercuric acetate (PMA) were made up as 10 mM stocks in ethanol and DMSO, respectively, using Viton or 4H gloves (Fisher Scientific) and proper precaution. For some experiments, PMA was used in place of CH_3HgCl. PMA is an alternative substrate for the MerB enzyme, kills wild-type and *merB* plants at approximately the same concentrations as CH_3HgCl, and is

preferred to CH_3HgCl for experimental purposes, as it is less volatile and less toxic to humans.

In vivo assay for conversion of organic mercury to Hg(0). Individual plants were weighed then immersed in a 50 mM phosphate buffer containing 25 µM phenylmercuric acetate. Plants were incubated in the assay buffer and sampled for evolved Hg(0) every 2 minutes over a 10 minute duration. A mercury vapor analyzer (Jerome 431-X; Arizona Instruments) was used to quantify Hg(0) at each time point. Instrument readings were converted to pg or ng Hg(0) on the basis of a standardization procedure carried out prior to each set of assays [22]. Mercury evolution rates for individual plants were calculated as per minute averages of pg Hg(0) / mg fresh weight plant tissue. The numbers of *merA/merB* plants averaged per line were: *merA/merB-1* = 10; *merA/merB-2* = 6; *merA/merB-3* = 6; *merA/merB-4* = 6; *merA/merB-5* = 8; *merA/merB-6* = 4. Three wild-type plants, 9 *merA* plants, and 9 *merB* plants were assayed as controls. Because F_2 lines represent segregating Mendelian populations, a proportion of the plants within each *merA/merB* line did not convert PMA to Hg(0). Volatilization rates for *merA/merB* lines represent averages of plants that had measurable positive enzymatic activity over background.

Quantitative western blot analysis and regression analysis. In a separate analysis, seventy-eight plants chosen randomly from six *merA/merB* lines were sampled for Hg(0) volatilization following the method described in the previous section. Sixty-two of these plants were used to isolate protein. Individual plants were placed in minicentrifuge tubes, dipped in liquid nitrogen, and ground with plastic pestles. Protein was extracted by adding 75 µl of plant protein extraction buffer [15], vortexing, spinning

down for 2 min. at 10,000 rpm, transferring supernatant to a new tube, and spinning down a second time. 100 to 200 µl of supernatant was withdrawn for each sample and combined with an equal amount of 2X SDS Sample Buffer [15]. Protein samples (30 µl) were run on a series of polyacrylamide gels. After transferring the proteins to PVDF membranes (Immobilon-P, Millipore), each membrane was cut into three horizontal strips (>90 kDa, 35-90 kDa, <35 kDa), enabling us to probe separately for phosphoenolpyruvate carboxylase (PEP; 110 kDa), MerA (66 kDa), and MerB (24 kDa), respectively. The three filter strips were reacted with a PEP-specific antibody (Rockland, Inc.), a MerA-specific monoclonal antibody, MAb 11F9 [25], and a MerB-specific monoclonal antibody, MAb 2H8 [15], respectively. Signals were visualized using chemiluminescence (Amersham Life Science, Inc.).

Band densities for PEP, MerA, and MerB were determined using a densitometer and ImageQuant software (Molecular Dynamics, Inc.). To control for variations in luminescence among different gels, MerA and MerB sample densities were first standardized by dividing each value by the density of a constant amount of MerA (0.6 µg purified protein) or MerB (0.6 µg purified protein), respectively, loaded into separate lanes of each gel. To control for variations in loading or electroblotting efficiency among experimental samples, all samples were corrected for the relative amount of PEP protein detected in the same lane. PEP is a constituitively expressed protein that should be equivalent among all samples. The amount of PEP in each experimental sample was divided by the amount of PEP in a standard extract that was run in one lane on each gel. For the bar graphs presented in Figs. 4D, E, MerA and MerB values were normalized to

the highest level. Regression analysis was performed by Statview V 5.0.1 (SAS Institute, Inc.).

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Table 1. Effects of Mer enzyme expression levels on Hg(0) evolution rate.

ANOVA Table	DF	SS	MS	F-Value	P-Value
Regression	3	4.518	1.506	10.537	<.0001
Residual	44	6.289	.143		
Total	47	10.807			
Regression Summary			Model parameters	Coefficient	P-value
Count[1]	48 plants		log(MerB)	1.142	< 0.0001
r^2	.418		log(MerA)	-0.163	0.4119
			log(MerB) • log(MerA)	-0.834	0.001
			y-intercept	2.540	< 0.0001

[1]Plants without measurable levels of MerA or MerB were omitted from this analysis because log(0) is undefined.

Figure 1. Mercury biogeochemical cycle and methylmercury biomagnification. The arrows in this simplified diagram show the major interconversions of mercury in the environment. Elemental (Hg(0)) and sulfur-bound mercury (RSHg) are slowly converted to free ionic mercury (Hg(II)), which is a substrate for bacterial methylation. The product of this reaction, methylmercury, is a far more serious environmental threat than other forms of mercury due to its tendency to enter the aquatic food chain and biomagnify. It has been estimated that methylmercury bioconcentrates in fish six to seven orders of magnitude above concentrations found in polluted waters and constitutes 90 to 100% of their total mercury content. Ionic mercury, by comparison, bioconcentrates less efficiently and is more readily eliminated by the renal system.

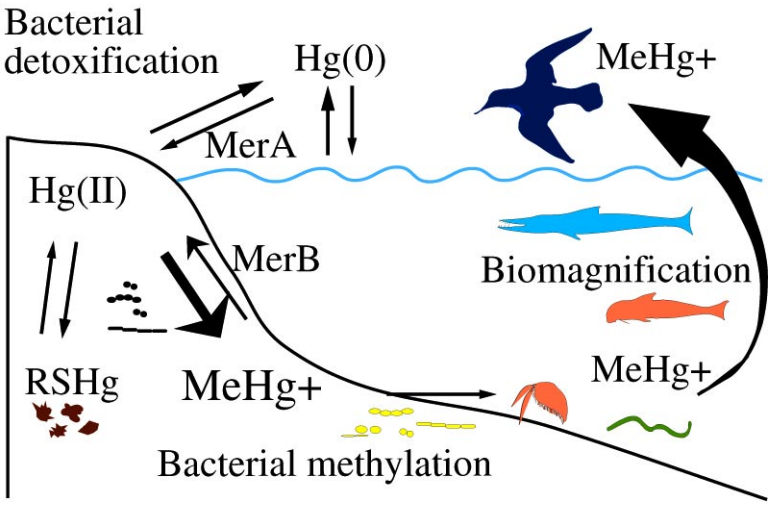
Bacterial
detoxification
Hg(0)
MeHg+
MerA
Hg(II)
MerB
Biomagnification
MeHg+
RSHg
MeHg+
Bacterial methylation

Figure 1.

Figure 2. Transgenic plants on organic mercury. A-D. A comparison of the growth of wild-type, *merA*, *merB*, and *merA/merB Arabidopsis thaliana* plants. Seeds from the wild-type (RLD ecotype), *merA9-1* [22], *merB-4* [15], and *merA/merB-1* plant lines were aligned in a horizontal array and grown vertically to observe root development. Growth medium contains (A) 0 μM / 0 ppm (B) 1 μM / 0.2 ppm (C) 5 μM / 1 ppm (D) 10 μM / 2 ppm CH_3HgCl as indicated. Plants were grown at 26 ^oC with 16 h light / 8 h darkness for 3 weeks. **2E, F.** Organic mercury resistance phenotypes of wild-type and six independent *merA/merB* plant lines. In order to compare plant lines for resistance to organic mercury, wild-type and *merA/merB* lines 1-6 were germinated side-by-side on agarose substrate containing $^1/_2$X MS salts and 0 (E) or 5 μM (F) PMA. Plants were grown on vertically positioned plates at 26 ^oC with 16 h light / 8 h dark for 3 weeks. The organic mercury resistance phenotypes of the *merA/merB* lines appeared to correspond to their Hg(0) evolution rates (Fig. 3).

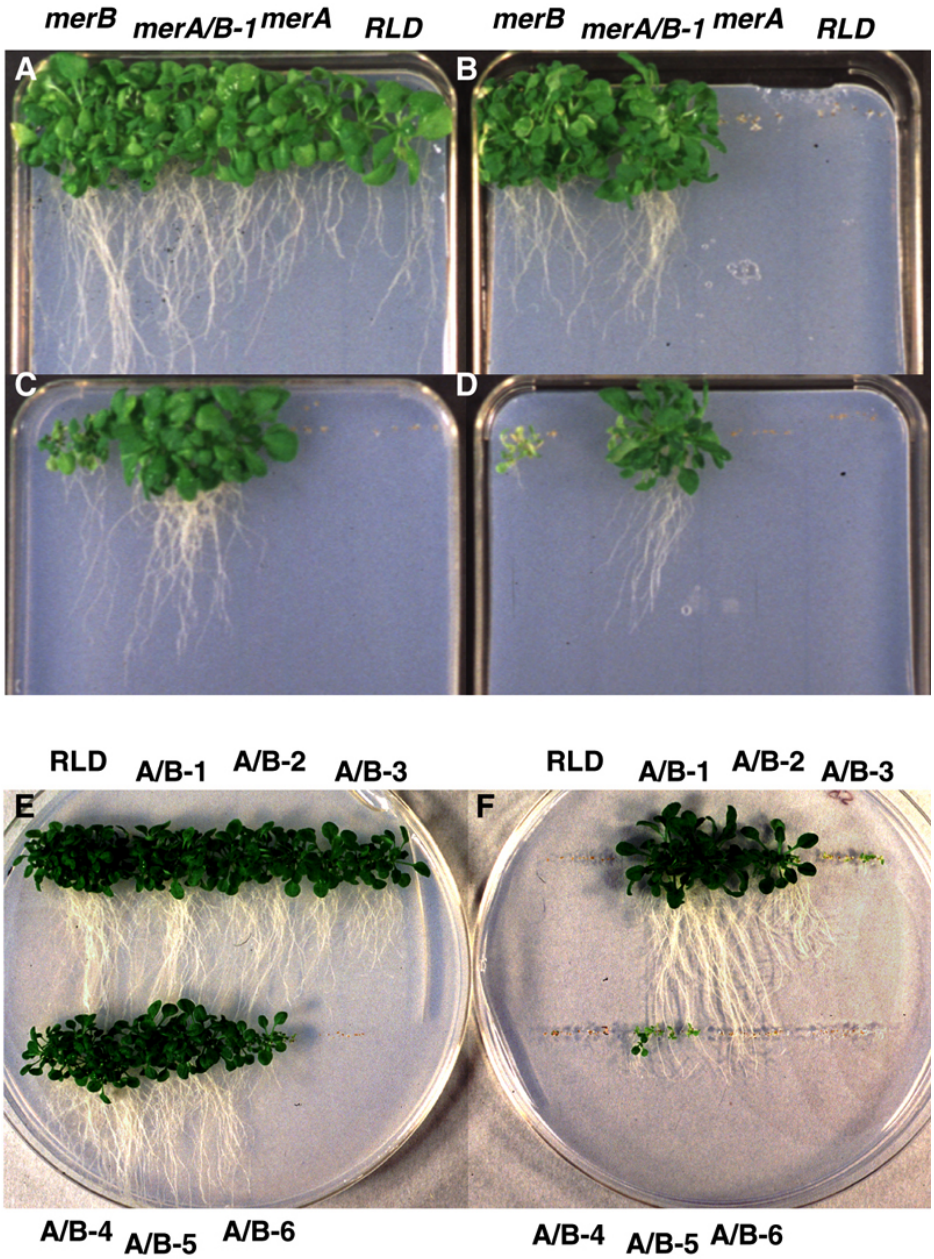
merB
merA/B-1
merA
RLD
merB
merA/B-1
merA
RLD
A
B
C
D
RLD
A/B-1
A/B-2
A/B-3
RLD
A/B-1
A/B-2
A/B-3
E
F
A/B-4
A/B-5
A/B-6
A/B-4
A/B-5
A/B-6

Figure 2.

Figure 3. Hg(0) evolution rates of wild-type, *merA*, *merB*, and *merA/merB* *Arabidopsis thaliana* plants. Individual plants ranging between 36 and 639 mg in wet tissue weight were removed from mercury-free growth medium and incubated at 24^{o}C in a sampling chamber filled with 1.5 ml of 25 μM PMA. At 2 minute intervals, the air in the chamber was evacuated and measured for gaseous Hg(0) with a Jerome 431-X Mercury Vapor Analyzer [22]. The assay was carried out for a duration of 10 minutes. Values reported in the bar graph in pg Hg(0) • mg $^{-1}$ plant tissue (fresh wt.) • min.$^{-1}$ represent per minute averages of 10 minute cumulative totals. Error bars represent the standard error for 3 to 10 plants. The values for RLD, *merA*, and *merB* were all equivalent to background levels which were less than 0.1 pg Hg(0) • mg $^{-1}$ plant tissue (fresh wt.) • min.$^{-1}$

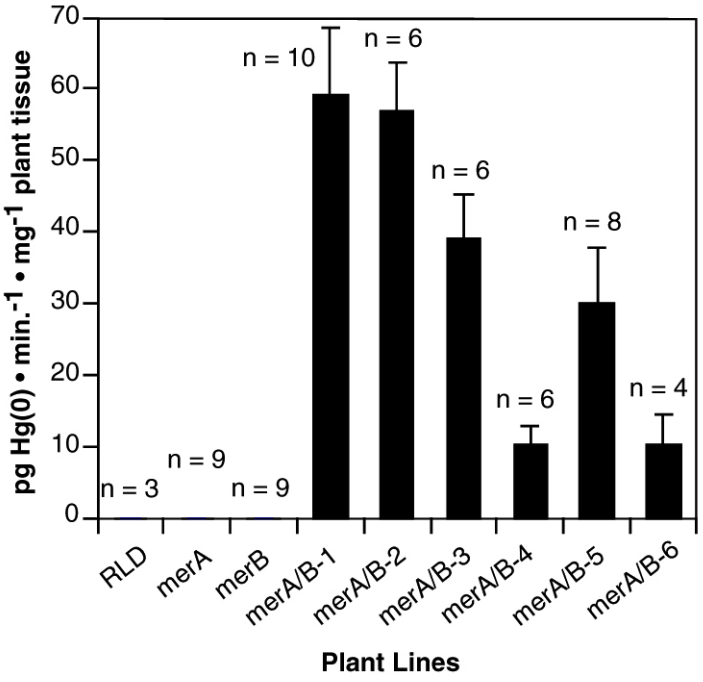
pg Hg(0) • min.-1 • mg-1 plant tissue
70
60
50
40
30
20
10
0
n = 3
n = 9
n = 9
n = 10
n = 6
n = 6
n = 6
n = 8
n = 4
RLD
merA
merB
merA/B-1
merA/B-2
merA/B-3
merA/B-4
merA/B-5
merA/B-6
Plant Lines

Figure 3.

Figure 4. Relationship between Mer enzyme levels and mercury volatilization rate. **(A-C)** Quantification of PEP control, MerA, and MerB protein levels. After recording an Hg(0) evolution rate, the levels of PEP, MerA, and MerB in each plant were determined by resolving total protein on SDS-PAGE, western blotting, and quantifying protein band densities. Fixed amounts of purified MerA and MerB protein and wild-type plant extract (PEP) were run as quantification standards. Blots were cut into three strips to probe separately for PEP (Fig. 4A), MerA (Fig. 4B), and MerB (Fig. 4C) with enzyme specific antisera (Experimental Protocol). MerA is proteolytically processed and, therefore, is present as two different molecular weight species. As shown, protein samples 2, 4, 6, and 7 do not contain both enzymes. Accordingly, the plants from which these four samples were isolated did not evolve Hg(0) when incubated in 25 μM PMA. This gel is representative of a series of gels used to correlate Hg(0) evolution with enzyme levels. Enzyme density values measured in this experiment were used in the regression analysis shown below. The sizes of standard proteins are indicated. **(D)** Effect of MerB protein levels on the Hg(0) evolution rate. The mean Hg(0) evolution rates for plants grouped by MerB expression levels are shown. B = normalized MerB expression levels. n = the number of plants in each group. **(E)** Effect of MerA protein levels on the Hg(0) evolution rate. See 4D.

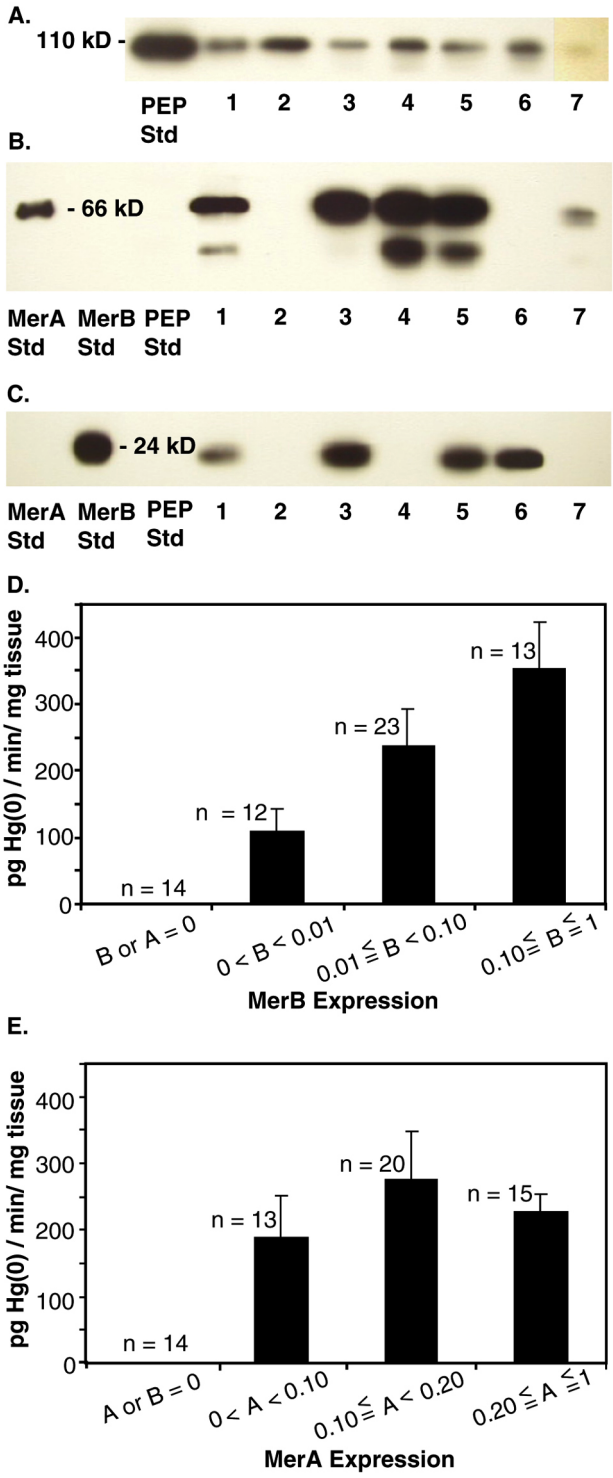
A.
110 kD -
PEP Std
1
2
3
4
5
6
7
B.
- 66 kD
MerA Std
MerB Std
PEP Std
C.
- 24 kD
D.
pg Hg(0) / min/ mg tissue
n = 14
n = 12
n = 23
n = 13
B or A = 0
0 < B < 0.01
0.01 ≦ B < 0.10
0.10 ≦ B ≦ 1
MerB Expression
E.
n = 14
n = 13
n = 20
n = 15
A or B = 0
0 < A < 0.10
0.10 ≦ A < 0.20
0.20 ≦ A ≦ 1
MerA Expression

Figure 4.

CHAPTER 4

TARGETED EXPRESSION OF MERB ENHANCES THE EFFICIENCY OF ORGANIC MERCURY DETOXIFICATION IN PLANTS [1]

---

[1]Scott P. Bizily and Richard B. Meagher. 2000.

To be submitted to Nature Biotechnology.

ABSTRACT

Methylmercury is an environmental pollutant that bioaccumulates in the aquatic food chain with potentially severe consequences for humans and other animals. In an effort to provide an affordable technique for removing this toxin in situ, we have been engineering plants to express the bacterial mercury resistance genes, *merA* and *merB*. These plants absorb organomercurials and convert them to Hg(0), a much safer and less reactive form of mercury. In vivo kinetics experiments suggested that inefficiencies in the diffusion of organic mercury to MerB might limit this reaction. In the interest of optimizing reaction kinetics, we have altered the intracellular sites of MerB synthesis, movement, and distribution so that the enzyme comes more readily in contact with its substrate. Using previously characterized plant targeting signals, we have tagged MerB both for secretion and for accumulation in the endoplasmic reticulum. Compared to plants with the cytoplasmically distributed wild-type enzyme, the new experimental lines degrade organic mercury at 10 to 100 times higher specific activity (rate per unit enzyme). The increase in reaction efficiency suggests either that (1) we have created a more efficient enzyme, (2) that organic mercury accumulates in the microenvironments (i.e. ER, Golgi, microvesicles, cell wall) in which we have targeted MerB, or (3) that these compartments provide more favorable reaction conditions (e.g. appropriate thiol acceptors).

Key Words: phytoremediation, mercury pollution, methylmercury, environmental engineering, subcellular localization, endoplasmic reticulum, secretion, *merA*

Introduction

Phytoremediation is an environmental engineering stategy in which plants are used to absorb and degrade toxic chemicals and either sequester or eliminate less toxic products. Current interest is motivated by the prospect of reducing the high financial and environmental costs of cleaning up polluted sites by traditional dredging, excavating, and landfilling methodologies. Plants have long been known to have an excellent capacity for binding metal ions and absorbing organic components of the groundwater in addition to growing on high concentrations of noxious chemicals. In theory, plants could act as valuable tools for filtering effluents, extracting chemicals from soil, and, potentially, converting toxins to less harmful products. Ongoing research is advancing the prospect of phytoremediation for metal pollutants including mercury, cadmium, lead, and a range of organic compounds such as TNT and TCE [1].

One approach has been to test common laboratory plant species for their natural abilities to break down pollutants. These efforts include the study of poplar trees for the degradation of trichloroethylene [2] and atrazine [3] and the study of sunflower and pea for phenanthrene [4]. A second approach has been to screen resistant plants from contaminated sites [5]. Although these strategies may utimately lead to the discovery and characterization of new degradative pathways, the novel systems being developed will not be fully manipulable until the enzymes (and genes) responsible have been identified.

An alternative approach is to make use of well characterized bacterial chemical resistance mechanisms, many of which are based on the enzymatic conversion of a toxic substrate to a less or non-toxic product [6-8]. A few labs have explored using bacterial genes for phytoremediation, and, in the past five years, have successfully engineered

plants to degrade methylmercury [9,10], reduce ionic mercury [11,12], chelate aluminum ions [13], and decompose nitroaromatic explosives [14]. Manipulable transgenes also offer the potential to fine tune enzyme concentrations, target gene products to specific tissues, and modulate subcellular expression patterns. Given that our ultimate objective is to provide maximally efficient tools for processing pollutants, our ability to modify these delivery parameters is of critical importance.

Recently, we have studied the effects of MerB (organomercurial lyase) and MerA (mercuric reductase) concentrations on PMA (phenylmercuric acetate) degradation in live *Arabidopsis thaliana* plants [10]. Our results described a positive, though non-linear, relationship between [MerB] and the rate of PMA to Hg(0) conversion

MerB

$$\mathbf{R\text{-}CH_2\text{-}Hg^+ + H^+ \dashrightarrow R\text{-}CH_3 + Hg(II)}$$

MerA

$$\mathbf{Hg(II) + NADPH + H^+ \dashrightarrow Hg(0) + NADP^+}$$

in which ten-fold increases in [MerB] result approximately two-fold increases in the conversion rate. By contrast, the rate at which Hg(II) is reduced to Hg(0) is linearly correlated with *merA* expression [11]. This suggested to us that the organomecurial substrate may not diffuse efficiently to the MerB enzyme. We propose two mechanisms that would cause this problem. For one, the cell wall and cell membrane present semipermeable barriers to methylmercury ions that are likely bound by free thiol ligands at the cell periphery. As a result, the toxin may accumulate in the cell wall and diffuse slowly into the cytoplasm. Secondly, it is feasible that methylmercury is partitioned into microenvironments inside the cell.

A recent study of the uptake of mercuric chloride by human fetal hepatic cells shows that Hg(II) accumulates at higher concentrations in mitochondria and the nucleus than in the cytosol [15]. This may relate to the presence of free thiol or imino-nitrogen ligands in these particular cell structures. It could also reflect abberant cellular processing in which Hg^{+}-S-X complexes are mistaken for metabolites and transported into the nucleus and mitochondria. There is precedence for the abberant processing of methlymercury in the transport of CH_3-Hg-Cys complexes across the neural cell membrane, a mechanism that, in part, accounts for the extreme neurotoxicity of methylmercury [16].

At this time, there are no data showing the precise localization of organic mercury in specific subcellular compartments. We suspect that, at the subcellular level, methylmercury adopts a non-uniform distribution due either to a misrouting mechanism or, alternatively, to natural pathways that cells use to process hydrophobic molecules. These pathways make use of the endoplasmic reticulum as a locus for accumulating and solubilizing the chemicals and consist of ER-resident enzymes such as the family of cytochrome P450's [17-20]. If methylmercury were shunted into organelles, such as mitochrondria and chloroplasts, the nucleus, or the endoplasmic reticulum while MerB were expressed primarily in the aqueous environment of the cytoplasm, then substrate and enzyme would not overlap, resulting in much slower binding kinetics than are observed in vitro.

Experiments with MerB tobacco cell cultures (Fig. 1) confirmed that the wild-type bacterial form of the enzyme localizes to the cytoplasm. In order to improve binding kinetics, we modified *merB* for (1) secretion of the protein, (2) accumulation of the

protein in the endoplasmic reticulum, (3) transit into mitochondria, and (4) transit into chloroplasts. We created four constructs, tested them in bacteria, transformed plants, and analyzed the effects of each construct independently in regard to mercury resistance and organic mercury degradation. In this chapter we compare *Arabidopsis thaliana* lines with cell wall (i.e. secretion) and endoplasmic reticulum-targeted versions of MerB to plant lines that accumulate MerB in the cytoplasm.

Results

Immunolocalization of MerB in cultured tobacco cells. *Nicotiana tabacum* plants were transformed previously with *merB* (unpublished data). Before considering options for modifying the intracellular distribution of MerB, it was necessary to establish how the wild-type form is distributed in plant cells. Initially, tobacco cells were used because they are large, easy to maintain in culture, and easy to prepare for microscopy. Fixed cells were reacted with a polyclonal anti-MerB antibody and an FITC-linked anti-rabbit secondary antibody. Confocal microscopy images reveal that MerB has a diffuse, cytoplasmic distribution and is excluded from the nucleus and vacuoles (Fig. 1). Wild-type tobacco cells had only a weak background signal due to autofluorescence.

MerB, CW-MerB, and ER-MerB confer resistance to phenylmercuric acetate (PMA) when expressed in *E. coli*. Two new *merB* constructs were created for targeting MerB protein using a strategy of overlap-extension PCR (Fig. 2). *CW-merB* contains an N-terminal fusion (signal sequence) to target MerB for secretion. In addition to the N-terminal signal sequence, *ER-merB* contains a C-terminal, ER-retention signal (KDEL) that is characteristic of a large number of ER-accumulated proteins. Before transferring

these constructs to plants, it was necessary to verify that the fusion products still had enzymatic activity and could be detected by the available anti-MerB antibodies. Enzyme functionality was verified by a metal ion disk assay in which 6 mm paper filter disks loaded with PMA were placed on top of freshly plated bacterial lawns (Fig. 3). The assay specifically measures metal resistance, in this case, a strong indicator that MerB is processing PMA.

Table 1.

Strain	*mer* genes	Note
pBS/pDU202/SK1592	Narrow spectrum *mer* operon	Negative control
pBS-*merB*/pDU202/SK1592	Narrow spectrum *mer* operon + wild-type *merB*	Positive control
pBS-CW*merB*/pDU202/SK1592	Narrow spectrum *mer* operon + modified *merB*	*merB* modified for plant cell wall targeting or secretion
pBS-ER*merB*/pDU202/SK1592	Narrow spectrum *mer* operon + modified *merB*	*merB* modified for plant endoplasmic reticulum targeting

The *E. coli* strain containing the wild-type form of *merB* was clearly the most resistant, although strains containing *CW-merB* and *ER-merB* were both substantially more resistant than the negative control. Zones of clearance in the presence of PMA for pBS-*CW-merB* /pDU202/SK1592 and pBS-*ER-merB* /pDU202/SK1592 were only 1 and 2 mm, respectively, larger than that for the wild-type *merB* strain.

Crude protein extracts from these strains were separated by SDS-PAGE, blotted, and probed with three independent anti-MerB antibodies, two monoclonals (MAb 2H8 and MAb10E2) and a polyclonal (PAb anti-MerB) (Fig. 4). Total protein loading was

equalized based on Coomassie blue staining. In order to make quantitative comparisons among MerB, CW-MerB, and ER-MerB on the same blot, we had to exclude the possibility that differences in protein levels could be attributable to differences in a single antibody's affinity for MerB, CW-MerB, and ER-MerB. The experiment implicitly assumes that all three antibodies do not recognize the same epitope and, therefore, that signal differences among the three proteins that remain consistent, regardless of which antibody is used, reflect real variations in protein levels. Western blots with MAb 2H8, MAb 10E2, and PAb anti-MerB showed a similar order of expression, although the polyclonal antibody appeared to react more strongly with the modified proteins, CW-MerB and ER-MerB, than the monoclonals. MerB was expressed at the highest level, followed by CW-MerB, and ER-MerB. Differences in expression level probably relate either to differential message or protein stability in *E. coli*.

PMA resistance phenotypes of plants expressing modified forms of MerB. Five independently isolated plant lines (T_2 generation) containing each new transgene in a constant *merA* background, were analyzed for resistance to organic mercury (Fig. 5). The same 35S promoter/NOS terminator combination was used for each gene construct. Two wild-type *merA/merB* lines (abbr. AB-1 and AB-5) [10] and one *merA* line were used as positive and negative controls, respectively. AB-1 is the most resistant *merA/merB* line that we have thus far isolated. Plants were grown on standard germination plates in the absence of PMA and with 1 or 5 μM PMA. Lines with the *CW-merBpe* and *ER-merBpe* constructs varied greatly in their resistance to PMA as we had found earlier for a variety of wild-type *merA/merB* lines. CW-1, 2, 5 and ER-1, 2, 3, and 5 were as resistant as the positive controls, AB-1 and AB-5, at 1 μM PMA. At 5 μM PMA, CW-2, ER-1,

and ER-2 were comparable to the most resistant positive control line, AB-1. CW-5 and ER-3 were similar to AB-5, a positive control with approximately one-third of the organic mercury processing capacity of AB-1 [10]. The most resistant plant lines, CW-2, ER-1, and ER-2, grew as rapidly and appeared as healthy on 5 μM PMA as on mercury free control plates. AB-5, CW-5, and ER-3 appeared heathly though grew at least 50% slower. Other lines germinated on 5 μM PMA but were severly stunted, suffered bleaching, and did not survive past four weeks.

Analysis of plants for conversion of PMA to Hg(0). An organic mercury conversion rate was determined for each independently derived plant line by measuring gaseous Hg(0), the final product of the coupled MerA and MerB-catalyzed reactions. Groups of 10 seedlings from each line were immersed in 25μM PMA for a period of 10 minutes. Hg(0) emissions were sampled at 5 and 10 minutes and used to calculate a PMA to Hg(0) conversion rate for each sample (Fig. 6). AB-1 and ER-2 converted nearly 800 ng/Hg(0)/min./g fresh wt. tissue. CW-2 produced Hg(0) at approximately 50% of this rate though was more rapid than any of the other lines, including AB-5. AB-5, CW-5, ER-1, and ER-3 had rates close to 200 ng/Hg(0)/min./g fresh wt. tissue. With the exception of the highly resistant line, ER-1, conversion rates reflected resistance levels. We had observed a similar correlation between organic mercury detoxification and growth while studying *Arabidopsis thaliana* with a range of *merB* expression levels [10]. Surprisingly, ER-1 had a resistance phenotype that compared with lines that had two to four times higher volatilization rates.

Quantification of MerB, CW-MerB, and ER-MerB levels in transgenic lines. Thirty 2-3 week-old seedlings from each line were assayed for MerB levels in crude

protein extracts (Fig. 7). Equal amounts of protein for all *CW-merBpe* and *ER-merBpe* lines were loaded on SDS-polyacrylamide gels, transferred to polyvinylidene membranes and reacted with antibodies. PEP-carboxylase levels were also measured on these western blots as a general indicator of total protein loading [10]. Relative to these lines, ten times less crude protein was loaded for wild-type *merB* lines, AB-1 and AB-5, since these lines had much higher MerB levels. Blotted protein was probed with the anti-MerB polyclonal antibody, PAb anti-MerB, which detects MerB as a single band at ~30-32 kD. CW-MerB and ER-MerB both ran 1-2 kD higher than the wild-type protein, suggesting that the N-terminal signal sequence had not been cleaved post-translationally or that a cleaved protein had been glycosylated. AB-1 contained 3-5 times the amount of MerB as AB-5. CW-2 contained approximately 14% as much MerB as AB-5. ER-2 contained approximately 13% as much MerB as AB-5. ER-3 had approximately 3% of the MerB as AB-5. ER-1 had approximately 1% of the MerB as AB-5. CW-1, 3, 4, 5, ER-4, and 5 did not produce detectable levels of MerB in three repetitions of this experiment. MerB was undetectable in CW-5 despite having volatilization and resistance phenotypes similar to ER-3. ER-1 had a very low level of MerB compared to CW-2 and ER-2, reflecting ER-1's lower volatilization rate but not its high level of PMA resistance.

Subcellular immunolocalization of MerB, CW-MerB, and ER-MerB.

Seedlings from an *Arabidopsis thaliana* line containing *merA* but not *merB*, and the highly resistant AB-1, CW-2, and ER-2 lines were prepared for fluorescent labelling. Cells were labelled with Texas Red (Molecular Probes, Inc.; Eugene, OR) which can be visualized using a standard rhodamine filter set. Confocal laser settings were adjusted using cells from the *merB* line, AB-1, which shows strong expression of MerB in the

cytoplasm (Fig. 8B). The large oval spaces without signal are chloroplasts, which are abundant in leaf cells. Under equivalent laser parameters, cells from the negative control (*merA*) line showed no apparent signal or a diffuse glow that appeared to be autofluorescence. The line ER-2 showed strong staining but in a pattern that was entirely different from that observed for AB-1. The signal, we believe, is localized in vesicles that are components of the ER/Golgi network. However, electron microscopy will be required to confirm the precise location of the protein. TEM will also be used in future experiments to localize CW-MerB in the line CW-2 since we were unable to observe a consistant signal (data not shown). There was clearly no specific localization of CW-MerB at the cell wall. This is likely due to the fact that the cell wall is partially digested with cellulase and pectinase during preparation of the cells for immunolabelling.

Discussion

Methylmercury can be a major environmental hazard despite being present at only trace levels in soils, sediments, or water. The chemical properties of methylmercury cause it to biomagnify efficiently and accumulate at dangerous concentrations in humans and other higher order predators. In the interest of developing a plant-based, in situ remediation system for methylmercury, we have transferred the bacterial *merB* and *merA* genes to both model and crop plant species. Our earlier experiments demonstrated that plants require only MerB and MerA to take up and convert organic mercury to Hg(0) [9]. We showed, furthermore, that the rate of conversion is related to the concentration of MerB, though not in a linear fashion. Approximately ten-fold increases in [MerB] yield approximately two-fold increases in reaction rate [10]. We reasoned that slow diffusion of

the substrate to the enzyme may limit the reaction kinetics. We hypothesized, furthermore, that poor substrate delivery could be caused either by blockage at the cell periphery or by the partitioning of organic mercury and MerB into separate microenvironments inside the cell.

We do not currently know how organic mercury is distributed in cells that have been treated with the toxin nor where the toxin has its most potent effects. A variety of techniques (immunolocalization, photographic development, spectrophotometry) used for measuring inorganic mercury concentrations would not be easily adaptable to organic mercury or would not be able to distinguish between organic and inorganic mercury ions. We have taken a reverse approach, modifying *merB* with targeting information for secretion and for ER-retention. We reasoned, first, that it would be best to place the enzyme at the point of the plant's initial contact with methylmercury, in the cell wall and at the epidermal surface. In this way it could process the toxin before it damages the cell. As an alternative strategy, we decided to accumulate MerB in the endoplasmic reticulum, an expansive, membrane-rich network for protein transport. Our primary rationale was that the ER is used by the cell to process hydrophobic toxins and, therefore, might develop higher concentrations of organic mercury than other cell structures. Furthermore, there are many possible targets for methylmercury toxicity in the ER because it is the site at which new proteins are processed and folded. Unfolded peptides must form disulfide bonds to adopt proper conformations. Methylmercury may intefere with this process due to its strong affinity for thiol groups. It should be noted that since both of our targeting strategies direct MerB into the endoplasmic reticulum, we would not expect to observe mutually exclusive patterns of protein localization.

Information for directing proteins to the secretory pathway is encoded at the N-terminus. We fused a signal sequence on the N-terminus of *merB* that tags proteins for translocation into the endoplasmic reticulum to create *CW-merBpe*. In the absence of additional targeting information, the protein should be secreted into extracellular space (i.e. the cell wall or through the epidermis). Specifically, we selected a sequence of 21 amino acids encoded by the *Nicotiana plumbaginifolia* extensin (*ext*) gene that had previously been shown to direct targeting of an NPTII (neomycin phosphotransferase II) reporter protein in *Nicotiana tabacum* protoplasts [21]. To create a construct for ER localization, *ER-merBpe*, we combined this sequence with a generic ER retention signal, KDEL, that is encoded at the C-terminus of a large number of ER-targeted proteins, both in plants and animals. This sequence has previously been shown to promote the retention of secreted proteins in the ER [22,23]. Using standard transformation techniques, we transferred the new genes to *merA Arabidopsis thaliana* [11].

Our analysis of five CW-targeted lines and five ER-targeted lines, indicated that these plants had much higher specific activity than plants with cytoplasmic MerB. For example, the ER-targeted lines, ER-1 and ER-2, and one of the cell wall-targeted lines, CW-2, had resistance levels equivalent to the best wild-type *merA/merB* line. One of these lines, ER-2, volatilized an equal amount of Hg(0) as AB-1. ER-1 and CW-2 volatilized Hg(0) at approximately 25% and 50% the rate of AB-1. Remarkably, ER-1, ER-2, and CW-2 all had less than 5% of the MerB expressed in AB-1. Furthermore, lines such as ER-5, CW-1, and CW-5 showed resistance despite having undetectable levels of MerB. These results suggest three possibilities: (1) that the placement of MerB in the cell wall and/or endoplasmic reticulum improves enzyme-substrate reaction kinetics because

these cellular structures have high substrate concentrations (2) that these subcellular compartments provide a chemical environment that favors the release of Hg(II) from MerB (3) that CW-MerB and ER-MerB are, in fact, more efficient enzymes than MerB.

We had initially considered it far more likely that the N-terminal and C-terminal additions to MerB would disrupt proper functioning of the enzyme. While it is possible that these modifications may have generated an enzyme with improved activity towards organic mercury, we suspect that this is not the case. CW-MerB and ER-MerB are expressed at roughly 50% and 20% of the level of MerB in *E. coli*, which corresponds to decrements in resistance that we observe in bacterial disk assays. By contrast, plants lines (i.e. CW-2 and ER-2) that express CW-MerB and ER-MerB at 14% of the level of MerB in the line, AB-5, are more resistant to PMA than AB-5 and convert PMA to Hg(0) approximately 2-3 times as rapidly. To further confirm this train of logic, we intend to measure PMA to Hg(0) conversion rates for *E. coli* strains containing *merB*, *CW-merB*, *and ER-merB* on a *merA* background. This will enable us to compute specific activities for the three forms of organomercurial lyase in bacterial and compare them to the specific activities we observe in plants.

Tabel 2.

Plant Line	[PMA --> Hg(0)]$_{Rate}$ (See Fig. 6)	Normalized [MerB] (See Fig. 7)	Specific Activity
AB-1	785	2.00 - 7.00	112 - 393
AB-5	260	1.00	250
CW-2	408	0.14	2914
ER-1	167	0.01	16700
ER-2	763	0.13	5869
ER-3	249	0.03	8300

Plant lines with CW-MerB and ER-MerB had 10 to 100 times higher specific activity [PMA --> $Hg(0)_{rate}$/ normalized enzyme concentration] towards organic mercury than lines with cytoplasmically expressed MerB. The fact that *CW-merBpe* and *ER-merBpe* lines did not significantly differ in their mercury processing capabilities argues that it is not secretion but rather localization of MerB in the secretory pathway (ER and Golgi) that explains the large increase in specific activity. The ER could be a favorable environment for MerB catalysis either because it is a compartment in which organic mercury is concentrated or because the biochemistry of the ER may differ from that of the cytoplasm in a critical way that enhances specific activity. For example, MerB requires a thiol acceptor in order to release Hg(0) and convert back into a reactive conformation. Different thiol acceptors, furthermore, appear to be more or less effective at releasing Hg(II) from the enzyme. The high specific activity observed in the ER may, by this rationale, be due to high concentrations of a particularly good thiol acceptor such

as free cysteine. Further experiments will clearly be required to explain the high specific activity observed in CW-MerB and ER-MerB lines.

Our highest priority, however, will be to confirm localization patterns of CW-MerB and ER-MerB using electron microscopy and immunogold tagging. Our results using immunofluorescence suggest that ER-MerB may, in fact, localize to the endoplasmic reticulum but were inconclusive concerning CW-MerB. We also intend to test for the secretion of CW-MerB by performing an immunoassay on hydroponic medium in which CW-*merB* plants have been grown. We especially hope to develop a suitable technique for measuring concentrations of organic and ionic mercury ions in subcellular microenvironments. Ultimately, however, our focus on MerB catalysis is motivated by our interest in creating an optimal tool for detoxifying organic mercury and remediating contaminated soils. In order to maximize the specific activity of the enzyme and minimize toxicity-related effects, it may be beneficial to localize MerB in several structures at once: the cell wall, the ER, mitochondria, chloroplasts, vacuoles, and the nucleus. Future work in our laboratory will explore these possibilities.

Experimental Protocol

Immunolocalization of MerB in cultured tobacco cells. Leaf disks from wild-type and *merB* tobacco were placed on regeneration medium [4.3 g/L MS salts (Gibco BRL; Grand Island, NY), 112 mg/L B5 vitamins (Sigma Chemical Co.; St. Louis, MO), 3% sucrose, 2 µg/ml NAA, 100 ng/ml kinetin, .8% Phytagar (Gibco BRL; Grand Island, NY)] to promote callus formation. Calli were removed, chopped finely with a razor, and placed in liquid medium (4.3 g/L MS salts, 112 mg/ml B5 vitamins, 3% sucrose, 2.7 mM

KH_2PO_4, 200 µg/L 2-4D) to support vegetative growth. The cell cultures was grown in the dark at 22°C with constant shaking (200 rpm). The medium was refreshed every two weeks. Before using, the cell cultures were filtered through cheesecloth to remove large calli. Cells were chemically fixed and labelled as described in Kandasamy et al. [24] except that the cells were probed with an anti-MerB PC primary antibody at 1:100 dilution for 16-18 hrs..

Modification of *merB* for altered patterns of subcellular distribution. A signal sequence (atg gga aaa atg gct tct cta ttt gcc aca ttt tta gtg gtt tta gtg tca ctt agc tta gct), derived from the *Nicotiana plumbaginifolia* extensin (*ext*) gene [21], was constructed de novo and fused to the 5' end of *merB* using overlap extension PCR. Five different oligonucleotides were synthesized. The first three, ext-1S, ext-2A, ext-3S overlapped each other by 18 nt's and encoded the N-terminal signal sequence as well as 5' restriction sites, an in-frame stop codon, a shine-delgarno sequence for bacterial expression, and sequence of six nucleotides typical of plant gene start sites. The final two primers, merB-A and merB-KA were both designed to overlap the 3' end of *merB*, and contained suitable 3' restriction sites. MerB-A was used to generate a construct with cell wall-specific targeting information while MerB-KA was used to build a construct identical but for the addition of the amino acids, (SE)KDEL, immediately in front of the stop codon. This short C-terminal sequence is typical of ER-retained proteins.

Initial rounds of PCR were performed with ext-3S and either merB-A or merB-KA as the primers and pBS-*merB* as the template. Appropriately sized fragments were gel-purified and used as templates for subsequent rounds of PCR with ext-2A and either merB-A or merB-KA as primers. The full length genes were completed in a third round

of PCR using ext-1S and each of the 3' primers. Final PCR products were gel-purified, digested with *Bam HI* and *Xho I* and ligated into digested pBluescript SK^{++} plasmids. These plasmids, named pBS-*CW-merB* and pBS-*ER-merB* were transformed into Top10F competent *E. coli* (Invitrogen, Inc.; Carlsbad, CA) by electroporation. Transformed colonies were identified by blue/white selection and grown up in 5 ml cultures. DNA was miniprepped and sequenced using the standard T3 and T7 primers to check for PCR errors. The plasmids, pBS-35S/*CW-merB*/NOS and pBS-35S/*ER-merB*/NOS were prepared by subcloning *Bam HI / Xho I* fragments into a Bluescript SK^{++} plasmid to which the 35S CaMV promoter and NOS 3' terminator had already been added. The entire promoter/coding region/terminator fragments were then subcloned into the pCAMBIA (Ray Wu, Dept. of Molecular Biology and Genetics, Cornell Univ., Ithaca, NY) vector by digesting at a *Sac I* site upstream of the promoter and a *Kpn I* site downstream of the NOS terminator. pCAMBIA-35S/*CW-merB*/NOS and pCAMBIA-35S/*ER-merB*/NOS were used for *Agrobacterium tumefaciens*-based plant transformations. The pCAMBIA vector confers kanamycin resistance to bacteria and hygromycin resistance to plants.

Construction of transgenic plants. *MerA Arabidopsis thaliana* plants were grown up and transformed using vacuum infiltration [25]. T_1 progeny were selected for hygromycin resistance and selfed. T_2 plants were used for all experiments described in this paper.

Bacterial resistance assays and western blotting. Bacterial strains were tested for resistance to PMA using a disk assay described previously [9]. The experimental strains were created by transforming the pDU202/SK1592 strain [26], which is resistant to

inorganic mercury, with pBS-*CW-merB* and pBS-*ER-merB*. Bacterial protein was isolated from these strains, as well as from positive and negative control strains, in the form of crude extract by sonication and mixed immediately with 2X sample buffer (4% SDS, 125 mM Tris-HCl pH. 6.7, 30% glycerol, 2% β-mercaptoethanol, 0.002% bromophenol blue). Western blots were probed with three separate antibodies to verify, first, that antibodies to MerB also reacted with CW-MerB and ER-MerB, and, second, that our antibodies did not have significantly higher or lower affinities for the modified proteins. Mab 2H8 and Mab 10E2 were used at 1 μg/ml concentrations while Pab anti-MerB was used at a 1:500 dilution. Primary reactions were carried out for 1 hr. after which the blots were washed 3 X 10 min. Secondary reactions with HRP-linked anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech.; Piscataway, NJ) were done at 1:2000 dilutions for 1 hr. Blots were washed 3 X 10 min. before development using a luminol-based kit (Amersham Pharmacia Biotech.; Piscataway, NJ). The blots were checked for equal loading by staining them directly with Coomassie blue after exposing them to film.

Plant resistance assays and western blotting. Organic mercury resistance assays were described previously and performed only with PMA [10]. Western blots were probed with the Pab anti-MerB antibody at 1:500 dilution for 1.5 hrs., washed for 3 X 15 min., probed with an HRP-linked anti-rabbit secondary antibody (Amersham Pharmacia Biotech.; Piscataway, NJ) for 1 hr., washed 3 X 25 min., and developed using a luminol-based kit.

In vivo enzyme assays. Measurements of PMA to Hg(0) conversion rates were done as previously described with the exception that samples consisted of 10 two week-old seedlings rather than individual plants [10].

Immunolocalization in plant cells. Seedlings from the AB-1, CW-2, and ER-2 lines were grown for 2-3 weeks on agarose plates then fixed by plunge freezing in liquid propane. Seedings were stored at –80^{o} C for 2-3 days then brought slowly to room temperature. The tissues were rehydrated by passing through an acetone series (100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 15%) and then into PME (50 mM Pipes buffer, 5 mM EGTA, 1 mM $MgSO_4$, 0.5% casein) containing a protease inhibitor cocktail (Cat. No. 1 836 170; Roche Diagnostics GmbH; Mannheim, Germany). Cells were dissociated by partially digesting the cell wall (approx. 10 min. digestion) in 1% cellulase, 0.1% pectolyase in the presence of protease inhibitors. Tissues were washed 1 X 5 min. in PME and 2 X 5 min. in PBS then macerated on poly-L lysine microscope slides to separate individual cells. Slides were allowed to air dry and were stored at 4^{o} C until further use. Prior to labelling, cells were treated with 0.5% Triton-X100 in PBS for 30 min., followed by 10 min. in methanol at -20^{o} C and a 10 min. wash in PBS. Cells were blocked with 5% BSA, 20% goat serum in TBS + 0.1% Tween-20 for 2 hrs. then labelled with anti-MerB PAb at 1:100 dilution overnight (16-18 hrs.). Cells were washed 3 X 15 min. in PBS, labelled with anti-rabbit Ig linked to Texas Red (Amersham Life Science; Cleveland, OH) at 1:100 dilution for 3 hrs., then washed again in PBS (3 X 15 min.). A mounting medium containing 0.1% para-phenylenediamene, 10% 0.01 M phosphate buffer in 0.15 M NaCl, 90% glycerol (v/v) was applied, and the slides were stored at -20^{o}C overnight to permit full diffusion of the anti-fading agent.

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Figure 1. Immunolocalization of MerB in transgenic cultured tobacco cells. MerB was localized using an anti-MerB polyclonal antibody and an FITC-conjugated secondary antibody. Cells were visualized with a Bio-Rad confocal microscope using 488 nm / 515 nm LP (excitation/emission) filters. The images shown are reconstructed stacks of 0.5 micron photographs. Under equivalent laser parameters, MerB was detected in the cytoplasm of transgenic cells **(B, C)** but not in wild-type cells **(A)**. MerB was clearly excluded from the nucleus and vacuoles and was distributed evenly throughout the cytoplasm except in a few cells **(C)** in which the protein aggregated at the nuclear membrane.

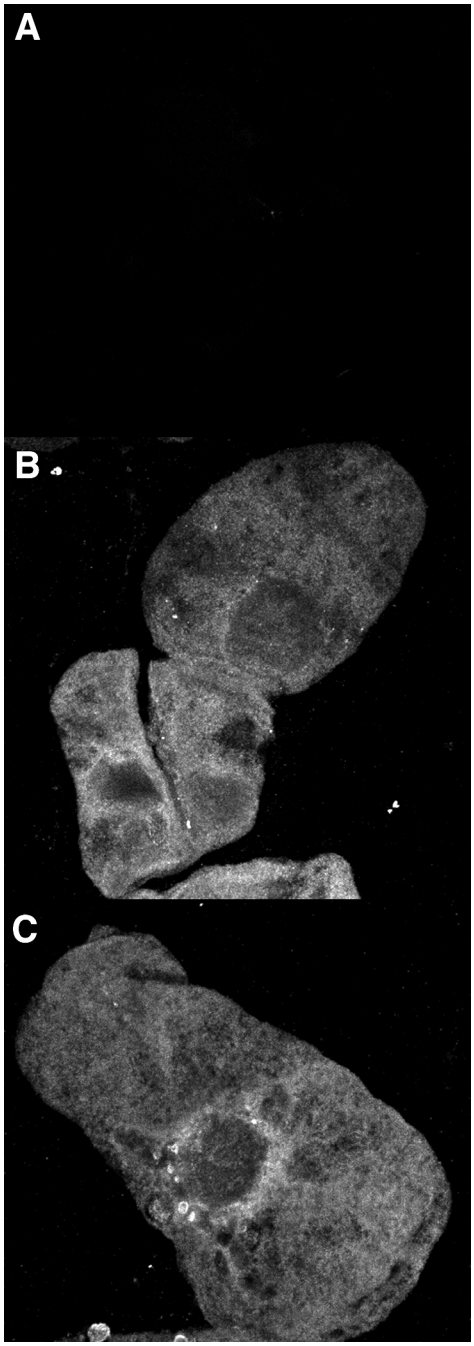

Figure 1.

Figure 2. Modification of *merB* for cell wall and endoplasmic-specific expression in *Arabidopsis thaliana*. The wild-type bacterial coding sequence of *merB* was altered in order to target the gene product to the cell wall and endoplasmic reticulum. Modified gene constructs, *CW-merB* and *ER-merB*, are referenced generically in the figure as *merBmod*. The same cauliflower mosaic virus 35S promoter / nopaline synthase terminator combination that drives expression of *merB* in lines AB-1 (*merA/merB-1*) and AB-5 (*merA/merB-5*) was used in the modified constructs. Overlap-extension PCR was used to add plant signal and retension sequences **(A)**. Full length modified genes were cut at *Bam HI* and *Xho I* and cloned into a pBluescript SK^{++} vector to create pBS-*merBmod* **(B)**. The same fragment was later subcloned into a pBluescript SK^{++} vector containing a CaMV 35S promoter / NOS terminator combination to create pBS-35S/*merBmod*/NOS **(C)**. The fragments 35S/CW-*merB*/NOS and 35S/CW-*merB*/NOS were cloned into the vector pCAMBIA for *Agrobacterium*-mediated plant transformations **(D)**.

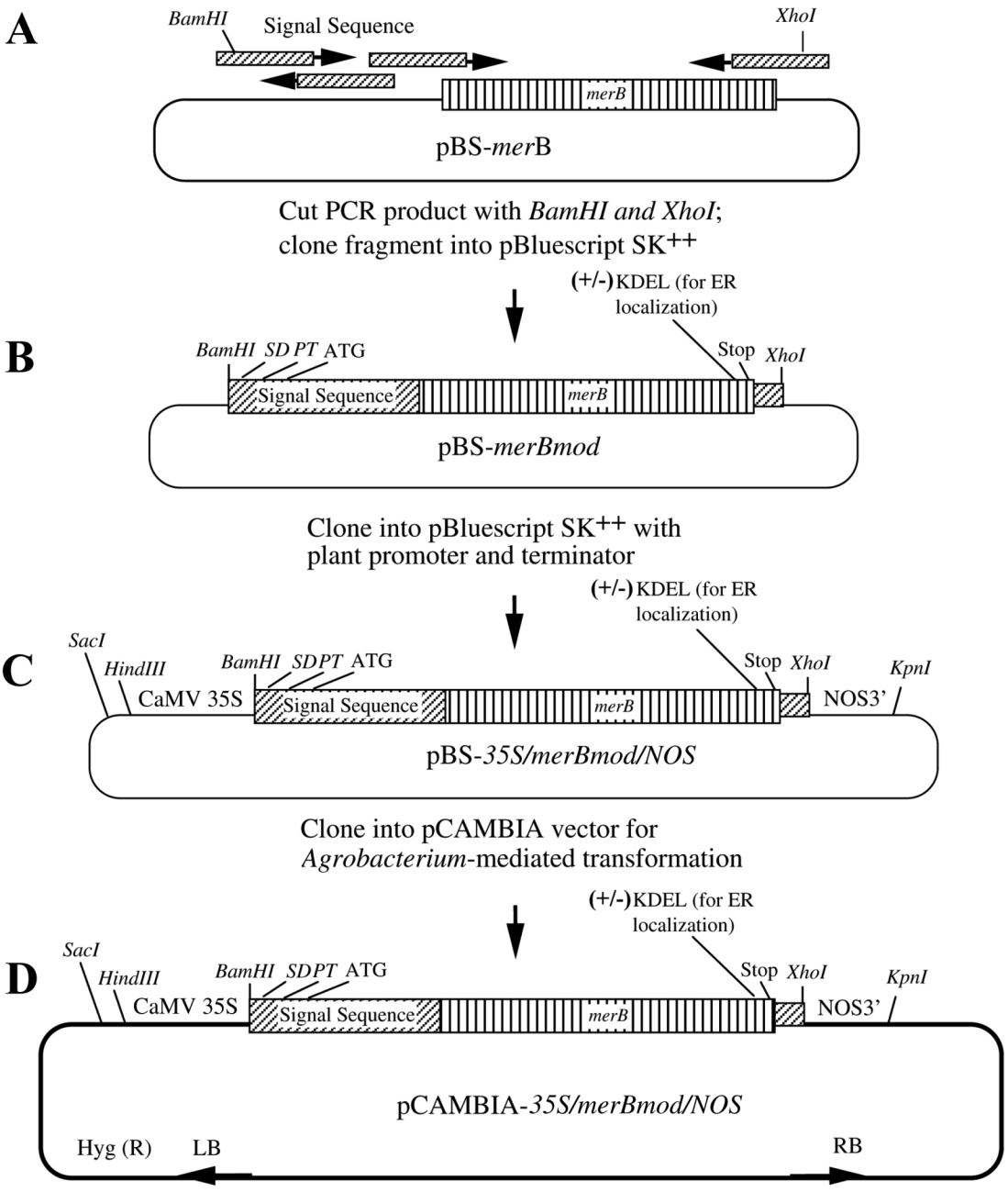
A
BamHI
Signal Sequence
XhoI
merB
pBS-merB
Cut PCR product with BamHI and XhoI;
clone fragment into pBluescript SK++
(+/-) KDEL (for ER localization)
B
BamHI SD PT ATG
Stop XhoI
Signal Sequence
merB
pBS-merBmod
Clone into pBluescript SK++ with
plant promoter and terminator
(+/-) KDEL (for ER localization)
C
SacI
HindIII
BamHI SDPT ATG
Stop XhoI
KpnI
CaMV 35S
Signal Sequence
merB
NOS3'
pBS-35S/merBmod/NOS
Clone into pCAMBIA vector for
Agrobacterium-mediated transformation
(+/-) KDEL (for ER localization)
D
SacI
HindIII
BamHI SDPT ATG
Stop XhoI
KpnI
CaMV 35S
Signal Sequence
merB
NOS3'
pCAMBIA-35S/merBmod/NOS
Hyg (R)
LB
RB

Figure 2.

Figure 3. CW-MerB and ER-MerB confer resistance to organic mercury. Bacterial disk assay s were used to verify the functionality of CW-MerB and ER-MerB in *E. coli* before transferring them to *Arabidopsis thaliana*. A diffusable source of organic mercury (PMA) is applied to a freshly plated bacterial lawn creating a concentration gradient of PMA. The proximity at which bacterial strains grow to the mercury source is a reflection of relative resistance. The zone of clearance is measured as the radius of the circular area in which the bacteria are unable to grow. Measurements are averages of at least six repetitions. Repetitions within strains varied by no more than 0.5 mm. A bacterial strain (pDU202/SK1592) with resistance to Hg(II) but not organic mercury was used as the recipient for an empty pBluescript SK^{++} plasmid [(-) *merB*] or a pBluescript SK^{++} plasmid containing wild-type *merB* [(+) *merB*], *CW-merB* [(+) *CW-merB*], or *ER-merB* [(+) *ER-merB*]. The positive control strain, (+) *merB*, has greatly enhanced resistance to organic mercury due to the expression of a native bacterial organomercurial lyase enzyme. Strains containing modified genes, *CW-merB* and *ER-merB*, are much more resistant to PMA than the negative control but moderately more sensitive than the positive control. This result may, in part, be explained by the fact that *CW-merB* and *ER-merB* appear to be expressed at a lower level in *E. coli* than *merB* **(Fig. 4)**.

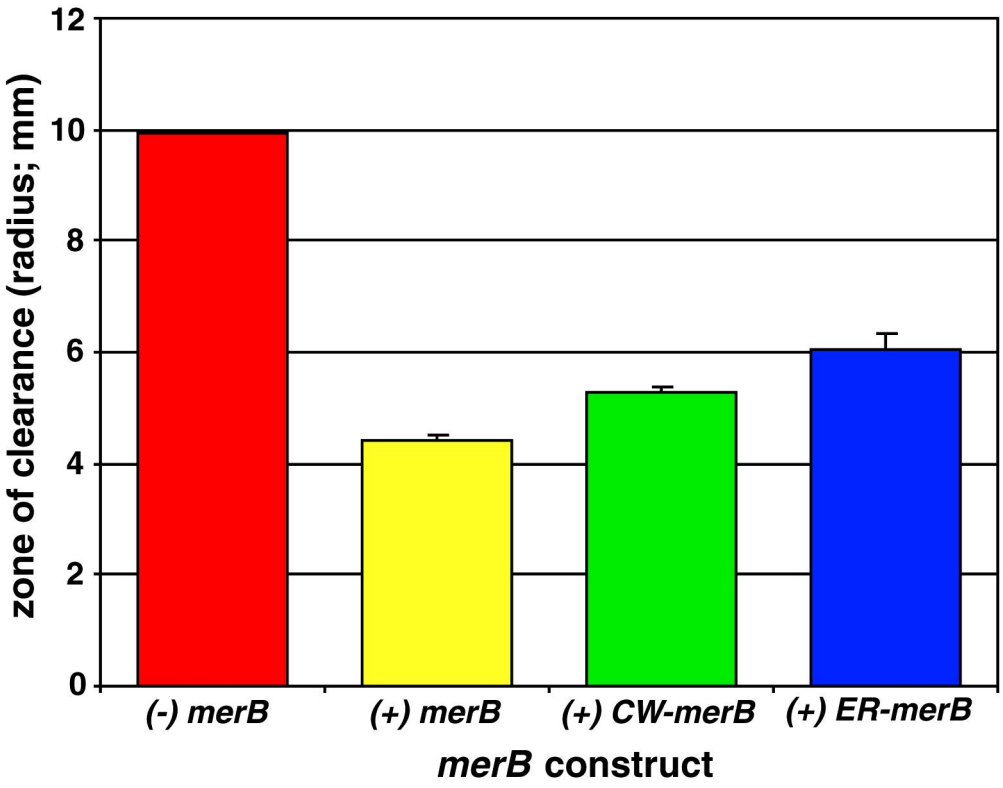
12
10
8
6
4
2
0
zone of clearance (radius; mm)
(-) merB
(+) merB
(+) CW-merB
(+) ER-merB
merB construct

Figure 3.

Figure 4. Western blot analysis of MerB, CW- MerB, and ER-MerB using three different antibodies. Bacterial extracts from strains containing pBS/*merB*, pBS/CW-*merB*, or pBS/ER-*merB* were separated on SDS-PAGE, blotted, and probed with an anti-MerB PC antibody and two anti-MerB monoclonal antibodies, MAb 10E2 and MAb 2H8. Anti-rabbit and anti-mouse IgG secondary antibodies were HRP-conjugated. Each of the antibodies detected both modified and wild-type forms of MerB. MerB gave the strongest signal, followed by CW-MerB and ER-MerB. This pattern was consistent regardless of the antibody used, suggesting that it may reflect actual differences in steady-state protein rather than variable immunogenicity. CW-MerB and ER-MerB ran at slightly higher molecular weights than MerB as would be predicted from their increased sequence lengths. Protein loading was equalized based on Coomassie blue staining. CW-MerB and ER-MerB were quantified and normalized relative to MerB levels in pBS/*merB*.

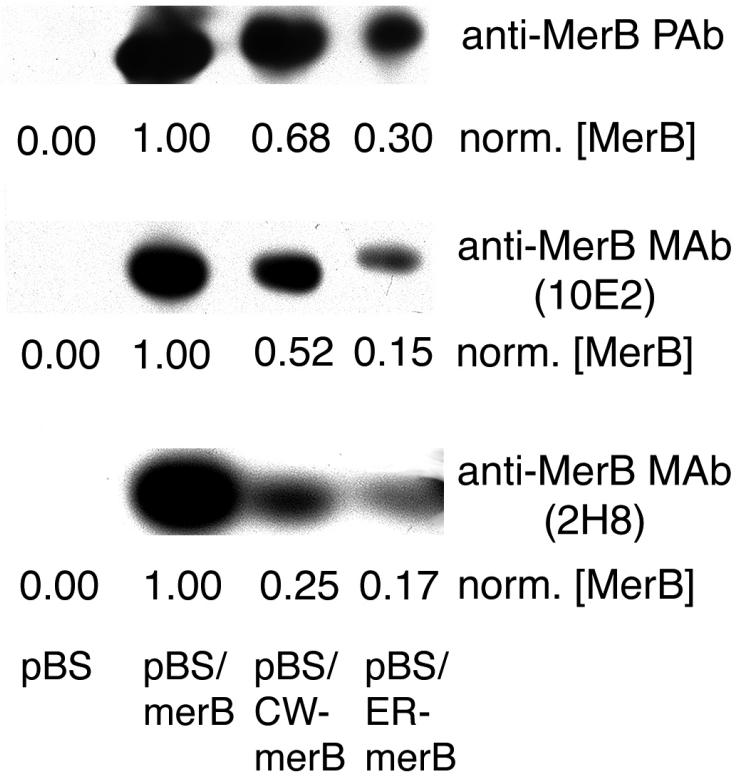
anti-MerB PAb
0.00 1.00 0.68 0.30 norm. [MerB]
anti-MerB MAb (10E2)
0.00 1.00 0.52 0.15 norm. [MerB]
anti-MerB MAb (2H8)
0.00 1.00 0.25 0.17 norm. [MerB]
pBS
pBS/ merB
pBS/ CW- merB
pBS/ ER- merB

Figure 4.

Figure 5. Resistance of *Arabidopsis thaliana merB, CW-merB, and ER-merB* lines to organic mercury. *Arabidopsis thaliana* seeds were germinated on 0.8% agarose plates containing standard plant growth medium with plant lines distributed as in **(A)**. The plates were dosed with either 0 µM PMA **(B)**, 1 µM PMA **(C)**, or 5 µM PMA **(D)**. Plants are shown at 16 days growth at 22^{o}C with 16 hrs. light / day. "Unrelated lines" have chloroplast-specific targeting of MerB and will be discussed in a future paper. Lines CW-2, ER-1, and ER-2 with cell-wall and endoplasmic reticulum-specific targeting signals had similar PMA resistance to the most resistant cytoplasmically expressed wild-type *merA/merB* line generated thus far, AB-1.

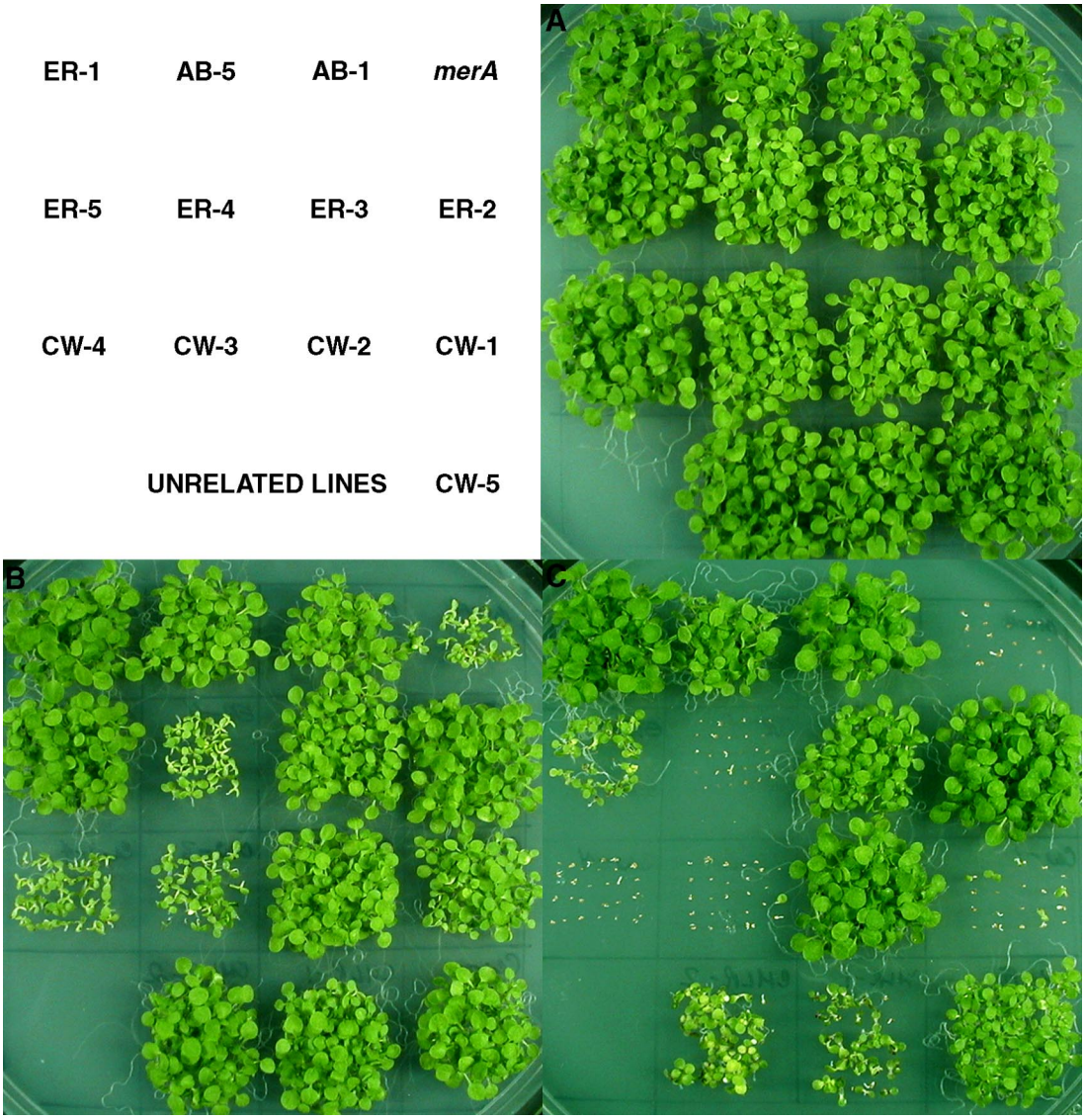
ER-1
AB-5
AB-1
merA
ER-5
ER-4
ER-3
ER-2
CW-4
CW-3
CW-2
CW-1
UNRELATED LINES
CW-5
A
B
C

Figure 5.

Figure 6. Organic mercury to Hg(0) conversion by selected *Arabidopsis thaliana merB, CW-merB, and ER-merB* lines. *Arabidopsis thaliana* plants from the lines shown in Fig. 5 were germinated on mercury-free agarose growth medium and grown for two weeks at 22^{o}C with 16 hrs. light / day. PMA to Hg(0) conversion rates were measured for transgenic lines using the following procedure. Ten T_2 seedlings were removed from the growth medium, weighed, and immersed in a liquid medium of 50 mM phosphate buffer, 25 μM PMA to begin a single assay. At 5 min. and 10 min. timepoints, the air in a partially sealed reaction chamber was evacuated and sampled for Hg(0) using a Jerome 431-X mercuy vapor analyzer (Arizona Instruments; Phoenix, AZ). Instruments readings were converted to ng Hg(0) based upon standards sampled at the beginning of each assay period. We report ng Hg(0) / min / g plant tissue (fresh weight) for each line as the average of three separate ten-seedling samplings. Among the lines expressing *CW-merB* and *ER-merB*, CW-2, CW-5, ER-1, ER-2, and ER-3 had the highest volatilization rates. The same lines were also the most resistant on 5 μM PMA **(Fig. 5C)**.

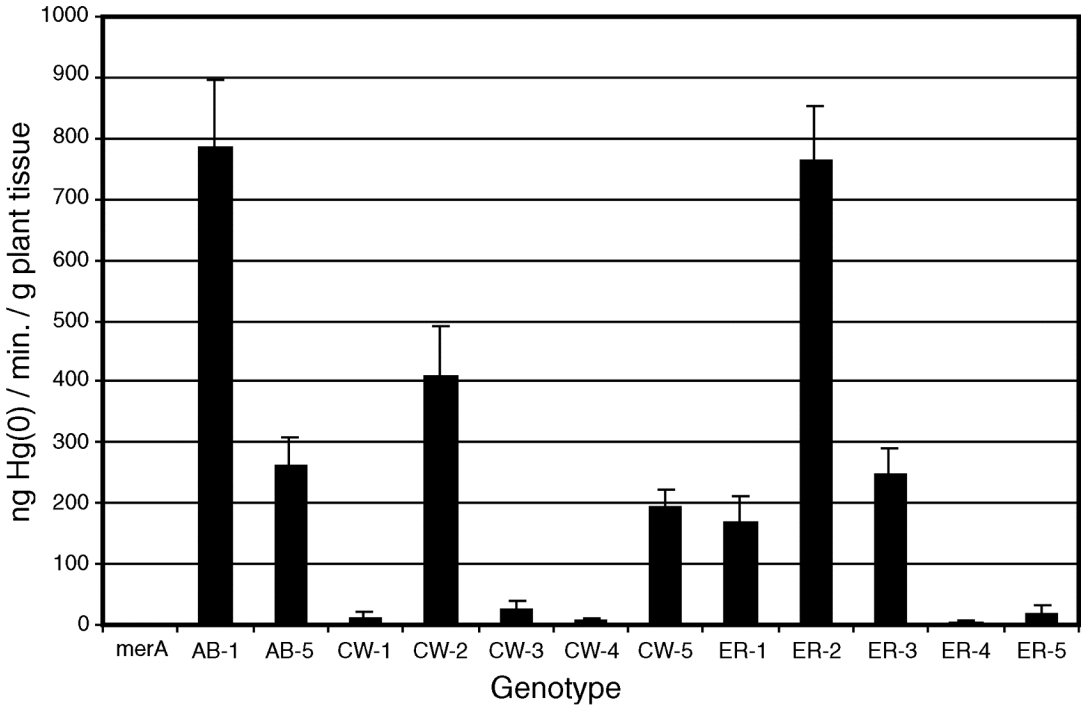
1000
900
800
700
600
500
400
300
200
100
0
ng Hg(0) / min. / g plant tissue
merA
AB-1
AB-5
CW-1
CW-2
CW-3
CW-4
CW-5
ER-1
ER-2
ER-3
ER-4
ER-5
Genotype

Figure 6.

Figure 7. Western blot analysis of MerB, CW-MerB, and ER-MerB expression levels in transgenic *Arabidopsis thaliana* lines. For each line, 30 one to two week-old seedlings were removed from mercury-free growth medium and ground in liquid nitrogen. Crude cell extracts were clarified by centrifugation and added to equal amounts of 2X Laemmli sample buffer (125 mM Tris pH 6.8, 4% SDS, 30% v/v glycerol, 2% β-mercaptoethanol, .002% bromophenol blue) containing a protease inhibitor cocktail (Cat. No. 1 836 170; Roche Diagnostics GmbH; Mannheim, Germany). Western blots were probed with the anti-MerB PAb and an anti-PEP-carboxylase PAb (Rockland, Inc.; Gilbertsville, PA) so that PEP-carboxylase could be used as a loading control. Proteins were detected by luminol-based immunofluorescence. Because lines AB-1 and AB-5 had a very strong MerB signal, crude protein loading for these lines was reduced ten-fold. In repeated experiments, strong MerB-specific bands were detected for the lines AB-1, AB-5, CW-2, and ER-2. Weak bands were detected for the lines ER-1 and ER-3. No band was detected for the line CW-5 which performed similarly to ER-3 in growth and PMA conversion assays. CW-MerB and ER-MerB were quantified and normalized relative to the MerB level in the line, AB-5.

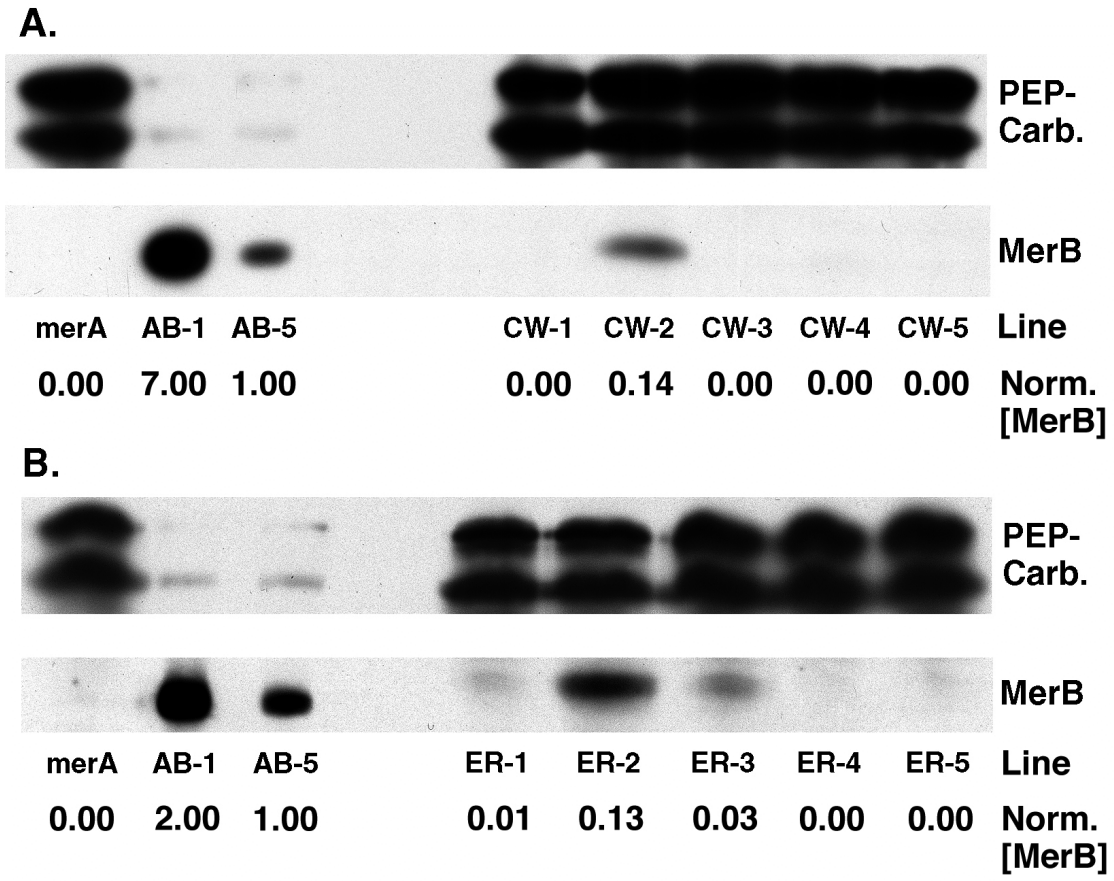
A.
PEP-Carb.
MerB
merA AB-1 AB-5 CW-1 CW-2 CW-3 CW-4 CW-5 Line
0.00 7.00 1.00 0.00 0.14 0.00 0.00 0.00 Norm. [MerB]
B.
PEP-Carb.
MerB
merA AB-1 AB-5 ER-1 ER-2 ER-3 ER-4 ER-5 Line
0.00 2.00 1.00 0.01 0.13 0.03 0.00 0.00 Norm. [MerB]

Figure 7.

Figure 8. Localization of MerB and ER-MerB in fixed *Arabidopsis thaliana* leaf cells using immunofluorescence. Cells were viewed using a Bio-Rad confocal microscope and 568 nm / 585 nm EFLP (excitation/emission) filters. MerB and ER-MerB were localized in the AB-1 and ER-2 lines, respectively, using an anti-MerB polyclonal antibody and a Texas Red-conjugated secondary antibody. The images shown are reconstructed from stacks of 0.5 micron photographs. Under equivalent laser parameters, MerB **(B)** and ER-MerB **(C, D)** showed different patterns of localization while cells from a (*merA*) control line **(A)** had only a faint background signal. MerB accumulates at high levels in the cytoplasm which can be seen interspersed around chloroplasts, the dominant subcellular structures in this cell type. ER-MerB, by comparison, accumulates in vesicles that we suspect are elements of the secretory pathway.

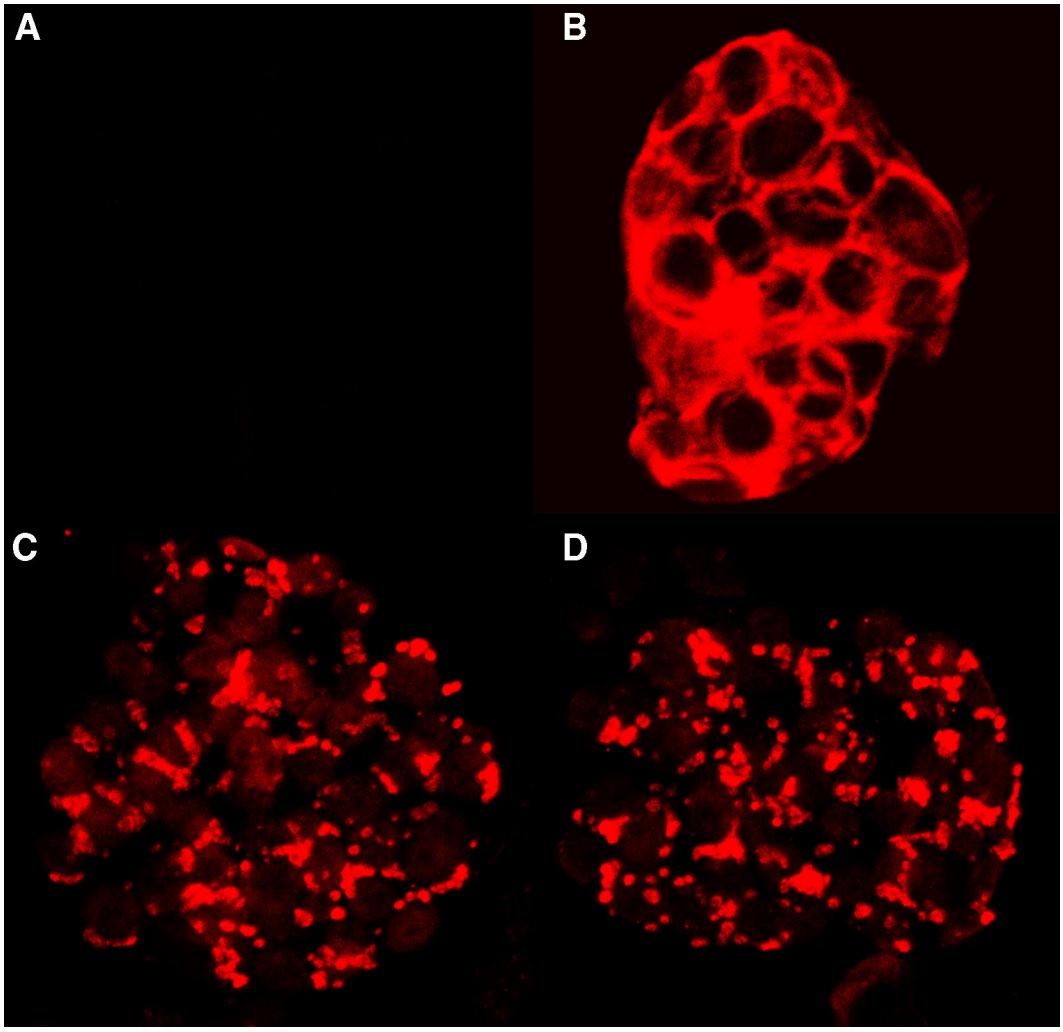

Figure 8.

CHAPTER 5

DISCUSSION

Mercury is considered one of the highest priority pollutants by the United States Environmental Protection Agency. Their recent "Mercury Study Report to Congress" is testimonial to the level of concern within the community of environmental scientists and policy-makers regarding the impact of mecurial pollutants on humans and whole ecosystems. Most mercury enters the environment in the ionic [Hg(II)] and elemental [Hg(0)] forms but can be converted in aqueous sediments to the neurotoxin, methylmercury. Methylmercury is an efficient biomagnifier and can reach dangerous levels in fish. According to the EPA, fishing advisories have been issued in 39 states due to high mercury levels. Projects are underway to dredge contaminated sediments from some sites and to prevent leaching into waterways.

The traditional approach for remediating sites contaminated with mercury, or other pollutants, is to excavate all soils and sediments that exceed regulatory limits for mercury concentrations. The soils are then transported to a lined landfill and capped over with an impermeable barrier. Disadvantages to this approach include high cost, a tendency to disrupt local ecosystems and promote leaching as soil is removed, and the questionable long-term stability of the landfills. Phytoremediation, a technology in which plants are used to extract and detoxify pollutants, has attracted attention as a cheaper, less disruptive alternative. The cleanup process occurs in situ, and labor/materials costs are greatly reduced compared to those incurred with excavation and landfilling procedures. The plants, furthermore, stabilize soils and promote ecosystem recovery.

Our laboratory has been developing a phytoremediation system for ionic and methylmercury pollution based on two genes found in bacteria. *MerA* (mercuric

reductase) and *merB* (organomercurial lyase) confer resistance to a range of organomercurial compounds, most notably methylmercury, by converting them to Hg(0). Elemental mercury is volatile at ambient temperatures and diffuses (out) through the bacterial cell membrane. We reasoned that, if plants were to manufacture MerA and MerB, organic mercury absorbed by the root system could be converted to Hg(0) which, could then be eliminated in the transpiration stream. We recognized that our strategy could fail at several levels:

1. Mercury might bind so tightly in the soil or growth medium that plants would not be able to extract it.
2. Plants might manufacture the enzymes at inadequate levels or produce modified or improperly folded forms.
3. Mercury might partition into plant tissues or subcellular compartments in which the enzymes are not localized.
4. Plant proteases might degrade the foreign-looking proteins.
5. Were the enzymes to function properly in the plant cytoplasm, the elemental mercury they produce could be re-oxidized by plant catalases and never leave the plant.

Most of these concerns were addressed by earlier work in our laboratory (see Rugh et al., 1996, 1998) in which *merA*-transformed *Arabidopsis thaliana* plants were shown to absorb Hg(II) and volatilize Hg(0). The media used were solid agarose and

hydroponics solution, not exact mimics of natural soils but good starting points. The next step would be to introduce *merB* to wild-type and *merA* plants.

Chapter 2 describes the analysis of *merB*-transformed *Arabidopsis thaliana*. To our surprise, we found that *merB* alone confers a high level of resistance to phenylmercuric acetate and methylmercury. MerB plants convert organic mercury into inorganic mercury [Hg(II)] but do not produce Hg(0). Although we did not have a biochemical test for this reaction, we later confirmed that it was occurring by observing the coupled reaction, PMA --> Hg(II) --> Hg(0), catalyzed by *merA/merB* plants. Immunolocalizations of MerB indicated that it is expressed cytoplasmically, suggesting that Hg(II) accumulates in this portion of the plant cell unless MerA is also present. In an non-compartmentalized environment with ideal diffusion characteristics, Hg(II) could be expected to have a higher toxicity than CH_3Hg^+ due to its ability to bind two ligands rather than one. Our experiment, however, indicates that, within the highly structured subcellular environment, CH_3Hg^+ is much more toxic than Hg(II). This may be related to the higher membrane permeability/mobility of CH_3Hg^+, which may permit it to flood sensitive organelles, or to a greater affinity of protective chelators (e.g. phytochelatins, metallothioneins) for Hg(II) compared to CH_3Hg^+.

We also found that independently transformed plants manufacture MerB protein at a range of expression levels, an effect due to differences in chromosomal position and copy number. We used this variation to study the impact of transgene expression levels on organic mercury resistance and on the rate of organic to elemental Hg conversion. As described in chapter 2, we found that MerB levels do not seem to greatly influence the resistance phenotype. This meant that one or two assumptions were incorrect: (1) that, as

far as CH_3Hg^+ is concerned, the plant cell could be treated as having ideal diffusion characteristics under which the enzyme concentration is directly proportional to the reaction rate; (2) that the reaction rate is positively related to the plant growth rate on CH_3Hg^+ substrate. The easiest way to distinguish between these two possibilities would be to test the first assumption by measuring the rate of CH_3Hg^+ (or PMA) loss or the rate of Hg(II) accumulation. Unfortunately, we did not have a technique for measuring organic or ionic mercury in vivo and in a timed kinetics assay. We approached the problem by using the coupled assay described in the following chapter.

Chapter 3 discusses our analysis of plant lines with both *merA* and *merB* genes. We used a coupled in vivo enzyme assay to measure Hg(0) produced by plants immersed in PMA. Plants containing both *mer* genes volatilized Hg(0) at a rapid rate while plants containing only *merA* or *merB* did not release detectable levels of Hg(0). This confirmed that both enzymes function in plants. We also found that lines from independent crosses (F_2: *merA* x *merB*) varied both in the rate at which they volatilized Hg(0) [$R_{Hg(0)}$] and in their resistance to organic mercury. Plants with higher volatilization rates had higher growth rates on organic mercury. An analysis of protein levels among the different lines revealed a correlation between [MerB] and [$R_{Hg(0)}$]. This is a non-linear correlation, however, in which ten-fold increases in [MerB] correspond to ~50-100% rises in the [$R_{Hg(0)}$]. [MerA], by comparison, did not correlate with [$R_{Hg(0)}$]. This is not surprising considering that MerA has a much higher catalytic efficiency than MerB and is the second enzyme in the organic mercury detoxification pathway.

In an in vitro experiment with ideal diffusion characteristics, the reaction rate is directly proportional to the enzyme concentration. In a coupled reaction with two

enzymes, one of which is much more efficient than the other, the reaction rate is proportional to the concentration of the less efficient enzyme. The absence of a linear relationship between [MerB] and [$R_{Hg(0)}$] suggests that some other process, such as the diffusion of the substrate to the enzyme, is limiting the reaction rate. We were aware that mercurials are not actively transported by plants and may not efficiently diffuse into the cells. Potentially, they bind to components of the cell wall, accumulate, and interfere with nutrient transport rather than disrupting internal components of the cell. However, we were also aware that both nutrient and toxic metals commonly adopt non-random subcellular distributions due to their own chemical properities, to the mechanisms that cells use to metabolize or process them, or to inadvertant processing based on molecular mimicry. If organomercurials acccumulated or were sequestered in specific cellular compartments, they would not efficiently react with MerB, which localizes to the plant cytoplasm.

To better pair MerB with microenvironments in which organic mercury accumulates we developed several targeting strategies, two of which are explored in the context of this dissertation. First, we targeted MerB for secretion (or cell wall deposition) by adding an N-terminal signal sequence that had previously been shown to route fused proteins into the secretory pathway. Second, we targeted MerB to the endoplasmic reticulum by fusing both the N-terminal fragment and a short C-terminal "retention" sequence, KDEL. Our analysis of plant lines transformed with modified versions of *merB* is discussed in chapter 4. After verifying the functionality and positive antigenicity of two modified proteins, CW-MerB and ER-MerB, we performed immunolocalizations to show that the proteins accumulate in the cell wall and endoplasmic reticulum,

respectively. Immunofluorescence (confocal microscopy) experiments seem to confirm an appropriate, ER-typical, pattern for ER-MerB, however were inconclusive for CW-MerB. This work is currently being followed up using TEM.

The modified proteins are clearly produced at a much lower abundance than the wild-type protein, which is expressed cytoplasmically. However, lines containing the modified constructs convert and grow on organic mercury at rates similar to those with the wild-type form. The increase (approx. 10 - 100X) in apparent specific activity (organic mercury conversion per unit enzyme) might be explained by higher substrate concentrations in the secretory pathway or by an ER-specific chemistry that favors some aspect of the enzymatic reaction. For example, the ER may contain high concentrations of a specific thiol acceptor that promotes the dissociation of the Hg(II) product from MerB.

A variety of studies could help to better address these questions. For example, if we could make point measurements of [CH_3Hg^+] or [PMA] in live cultured cells on an organellar level, it would allow us to determine whether the ER has high substrate concentrations. It may be possible to get rough estimates of subcellular substrate concentrations using either a fractionation protocol combined with standard chemical analysis or by using a methylmercury-specific tag (e.g. fluorescent molecule, radioisotope, etc.) and viewing cells with a confocal microscope. However, it could take a considerable investment of time to develop these techniques, and the data they yield might not be sufficiently precise. Along the same lines, it would be advantageous to have more information regarding the types and concentrations of free thiols in the plant ER and cytoplasm. With such knowledge, one could do informed in vitro biochemistry using

purified MerB, CW-MerB, and ER-MerB. This would allow for an assessment of the thiol dependence as well as the raw catalytic effiency of the three proteins.

While there is no immediate gain in the rate of organic mercury detoxification by targeting MerB to the secretory pathway, our studies do suggest that, if MerB were expressed at higher levels, we might observe considerable improvements. Additionally, there is a strong possibility that the combined expression of MerB in the secretory pathway and the cytoplasm could enhance the reaction rate. Our laboratory will pursue these possibilities in future work.