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Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *merA* gene

(toxic metals/heavy metals/pollution/sequestration/phytoremediation)

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ABSTRACT **With global heavy metal contamination increasing, plants that can process heavy metals might provide efficient and ecologically sound approaches to sequestration and removal. Mercuric ion reductase, MerA, converts toxic Hg^{2+} to the less toxic, relatively inert metallic mercury (Hg^0). The bacterial *merA* sequence is rich in CpG dinucleotides and has a highly skewed codon usage, both of which are particularly unfavorable to efficient expression in plants. We constructed a mutagenized *merA* sequence, *merApe9*, modifying the flanking region and 9% of the coding region and placing this sequence under control of plant regulatory elements. Transgenic *Arabidopsis thaliana* seeds expressing *merApe9* germinated, and these seedlings grew, flowered, and set seed on medium containing $HgCl_2$ concentrations of 25–100 μM (5–20 ppm), levels toxic to several controls. Transgenic *merApe9* seedlings evolved considerable amounts of Hg^0 relative to control plants. The rate of mercury evolution and the level of resistance were proportional to the steady-state mRNA level, confirming that resistance was due to expression of the MerApe9 enzyme. Plants and bacteria expressing *merApe9* were also resistant to toxic levels of Au^{3+}. These and other data suggest that there are potentially viable molecular genetic approaches to the phytoremediation of metal ion pollution.**

Heavy metals have reached toxic levels in the air, land, and water of many parts of the world (1, 2) and are an increasing problem in the sludge produced by industries and population centers (3). The wind-borne residue of volatile metals has contaminated land at great distances from smelting operations, compromising the economic value of the land (4). Areas where high concentrations of naturally occurring toxic metals including arsenic, cadmium, copper, cobalt, lead, mercury, selenium, and/or zinc are found such as the western United States (5) and Africa (6) are inhabited by scrubby, heavy metal-tolerant flora, which hyperaccumulate metal ion chelates (7). These hyperaccumulators offer exciting potential solutions for the phytoremediation of metal ion-contaminated sites (8, 9). However, an investigation of the genetic and biochemical complexity of hyperaccumulating systems has only just begun (10).

There are only a few systems of metal ion resistance and/or sequestration that are sufficiently characterized to be approached with molecular genetics. The phytochelatins in plants (11) and the metallothioneins (12) both contain a high percentage of cysteine sulfhydryl residues, which bind and sequester heavy metal ions in very stable complexes. Using alternative detoxification strategies, bacteria can electrochemically reduce a number of heavy metal ions and oxyanions to less toxic states (13). Mercury resistance in Gram-negative bacteria is encoded by an operon of genes (14), producing a mercury responsive regulatory protein; transport proteins that bind and transfer mercury into the cell; an organomercury lyase, which catalyzes the protonolysis of carbon mercury bonds releasing ionic mercury; and a mercuric ion reductase (MerA). MerA efficiently catalyzes the reduction of Hg^{2+} (15) according to the equation:

$$RSHg^+ + NADPH \rightarrow Hg^0 + RSH + NADP^+,$$

where $RSHg^+$ is a thiol-containing organomercurial salt. Nonionic and less toxic Hg^0 is then volatilized from the cell. Based on conserved amino acid sequence and function, MerA belongs to an evolutionarily diverse and ancient class of soluble, NADPH-dependent, FAD-containing disulfide oxidoreductases (15). Other members of this family can reduce a wide variety of organic compounds (16). *Escherichia coli* cells expressing MerA have weak reduction activity toward Au^{3+} and Ag^+ (17). It seemed possible that the *merA* gene could be used to develop plants resistant to Hg^{2+} and other metal ions (18). Lending support to this idea is the observation that yeast *Saccharomyces cerevisiae* expressing the *merA* gene are weakly resistant to Hg^{2+} (19).

Mercury pollution is a worldwide problem in aquatic environments, resulting primarily from its industrial use in bleaching operations (i.e., chlorine production, paper, textiles, etc.), as a catalyst, as a pigment in paints, and in the mining of gold. Its use in seed and bulb dressings directed against bacteria and fungi and in fungicidal sprays on fruit trees has introduced much of the mercury that contaminates agricultural land. If the original source of mercury is large enough even metallic mercury, Hg^0, becomes problematic, since biological systems can reoxidize it to Hg^{2+} at a low rate (20). Although the rate of release of mercury into the environment may have slowed in recent years (3), previously contaminated sites continue to leach large quantities of mercury into adjacent wetlands, waterways, and estuaries (21–23). The mercury that is not bound up in insoluble sulfur salts, $(RS)_2Hg$, tends to accumulate in invertebrates and fish as methylmercury (CH_3Hg^+), dimethylmercury [$(CH_3)_2Hg$], or other organomercury salts. The organomercury compounds are passed on rapidly to local bird, animal, and human populations with tragic consequences (24).

In this manuscript we describe the expression of *merA* in transgenic plants. Plants expressing only moderate levels of *merA* mRNA are resistant to levels of mercuric ion in the media that kill all control plants. These plants evolve significant levels of volatile mercury vapor relative to control plants. Possible schemes using metal ion reduction as part of a phytoremediation plan for metal-contaminated sites are discussed.

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Abbreviations: T-DNA, transferred DNA; OE, overlap extension.

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MATERIALS AND METHODS

Strains, Plasmids, Media, and Disk Assays. 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, which was described previously (25). The pBluescriptSKII$^+$ (pBSSKII) vector plasmid was obtained from Stratagene Inc. The *mer* operon-containing plasmids pDU202 and pPB111-47 have been described (26). pDU202 and pPB111-47 were mated into SK1592, selecting for the drug markers on these plasmids and using minimal medium to select against the parent bacterial strains and for SK1592. Each strain was then transformed to contain either pNS2 described below or the parent control plasmid, pBSSKII. All metal ion sensitivity filter disk assays were performed in the presence of ampicillin to maintain the pBSSKII plasmids and kanamycin or chloramphenicol to retain the *mer* operon plasmids when they were also present. Approximately 2×10^8 cells were plated in tryptone top agar onto tryptone plates. Five microliters of a freshly prepared metal ion stock solution, at the concentration indicated in Table 1, was deposited onto a 6-mm-diameter filter disk (Whatman 3M), and the filter disk was positioned on the freshly hardened top agar. The diameter of the zone of inhibition was measured after 16–20 hr of growth at 37°C, and the data reported are the average results of several replicates. The zone sizes among individual experiments varied by <0.5–1 mm for any of the data reported.

The Reconstruction of *merA* for Plant Expression. Flanking primers 5′S and 3′A and large mutagenic primers (99-mers) internal to the *merA* coding sequence, 282-312A and 307-339S (Fig. 1), were used to amplify the a and b halves of the *merA* gene with PCR from the *mer* operon in Tn*21*. The PCR amplification contained amplification buffer (Promega) with 6% dimethyl sulfoxide and 1.5 units of *Taq* polymerase (Boehringer Mannheim) and was carried out for 35 cycles (95°C for 1 min, 42°C for 1 min, and 72°C for 1 min). Without dimethyl sulfoxide we were unable to amplify significant amounts of the full-length wild-type *merA* gene with several commercial sources of *Taq* polymerase. Once gel purified, the a and b fragments (Fig. 1) were joined together in an overlap extension–PCR reaction (32) to produce the NS2 fragment. The same PCR conditions as above were used except that the extension time was 2 min and the reaction was primed with the external oligonucleotides 5′S and 3′A. The mutagenized fragment was cleaved in the flanking *Bam*HI and *Pst* I sites, ligated into the *Bam*HI/*Pst* I replacement region in the multilinker of plasmid pBluescriptSKII(−) (Stratagene) to make pNS2, and transformed into an Hg-supersensitive strain of *E. coli* and selected for ampicillin resistance. Most of the pNS2-containing strains grew well when replica plated on 100 μM $HgCl_2$. One transformant that grew particularly well on mercury was used in the further studies reported herein. DNA sequencing of the altered regions confirmed the intended *merApe9* gene and protein sequence with the exception of the two nucleotides shown in lowercase letters and the two underlined amino acids in Fig. 1*B*. These mutations resulted in conservative amino acid changes, Ile-311 → Val-311 and Ala-336 → Gly-336, and must have been introduced by the PCR amplification or the synthetic oligonucleotides (33).

The synthetic *Bam*HI site from the 5′S oligonucleotide and a *Xho* I site in the multilinker flanking the 3′ endo of *merApe9* in the pNS2 plasmid clone were cleaved, and the resulting fragment was ligated into the *Bam*HI/*Xho* I replacement region of the binary plant expression vector pVSTI (34).

Construction of Transgenic Plants. *Agrobacterium tumefaciens*-mediated transformation of embryos (35) induced in *Arabidopsis* (RLD var.) root explants resulted in a large number of independent transgenic shoots. These shoots (T0) were planted in soil without roots, fed topically, and allowed to go to seed (T1 seeds). Most transformation protocols result in one to three independent insertions of the transgene per plant (36), and thus normal Mendelian segregation cannot be expected. T1 plants from T1 seeds derived from independent root explants were self-fertilized to produce the nine independent T2 transgenic lines examined, nos. 1–9. PCR reactions performed on small tissue samples were used to show the presence of the transgene in T2 plants. Segregant lines derived from these were established during the T3 generation without selection. For example, three T2 seeds from line no. 1 were grown in soil to generate the three populations of T3 seed (1A, 1B, and 1C). Seeds, seedlings, and plants from the T2 and T3 generation were assayed using MS salts medium with sucrose (Life Technologies, Grand Island, NY). Heavy metals were incorporated into the medium after autoclaving.

Mercury Vapor Assays. Hg^0 is relatively insoluble and volatile and lost quickly from cells or media. Volatilized Hg^0

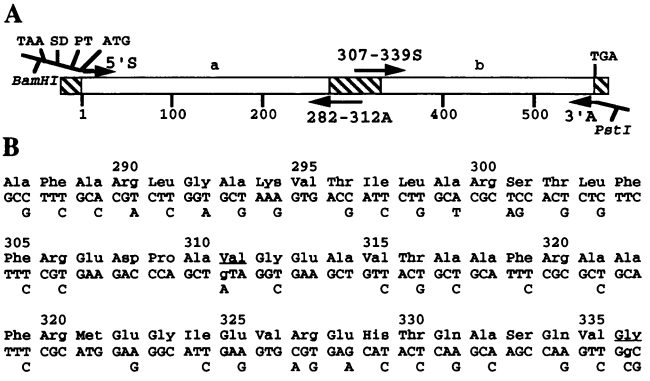

FIG. 1. Construction of the *merApe9* gene for efficient expression in plants and *E. coli*. (*A*) Strategy to mutagenize the *merA* gene. A map of the *merA* gene with codon numbers is shown. Overlap extension (OE)-PCR was used to mutagenize the *merA* gene and generate the synthetic *merApe9* sequence (see *Materials and Methods*). The a and b halves of *merApe9* were amplified in two separate PCR reactions, using pairs of sense (S) and antisense (A) mutagenic oligonucleotides, 5′S/282-312A and 307-339S/3′A, respectively. This allowed both flanking sequences and a large block of internal coding region to be modified (crosshatched areas). The 5′-flanking sequence primer, 5′S, had the 59-nt sequence 5′-CTAGAACTAG TGGATCCCTA GATCTAAGAA GGAACCACAA TGAGCACTCT CAAAATCAC-3′. This primer introduced the *Bam*HI site used to subclone the fragment and contained an in-frame stop codon, TAA, to end the translation of a β-galactosidase protein in *E. coli*; a consensus Shine–Dalgarno (SD) bacterial ribosome binding site, AGAAGG (27); a potentially important consensus sequence for plant translation (PT), AACCACA (28); and the first 19 nt of the *merA* coding sequence used to prime the forward PCR amplification of the a fragment. Slight changes from the original consensus sequences were made to avoid potential hairpin structures in the mRNA leader (29). The 3′-flanking sequence primer, 3′A, had the 36-nt sequence 5′-TATCGAATTC CTGCAGCCTCACCCGGCGCA GCAGGA-3′; included the *merA* stop codon, TGA; introduced a *Pst* I site; and ends in anticodons to the terminal six *merA* codons, which were used to prime the reverse PCR amplification of the b fragment. Both 5′S and 3′A primers lowered the G+C composition of the flanking sequences. The internal primers, 282-312A and 307-339S, altered 37 codons in a 54-codon region in the center of the gene (see *B*) and contained 18 nt of sequence overlap. The purified a and b fragments were joined into the intact *merApe9* gene by overlap extension PCR using the 5′S and 3′A primers. (*B*) The DNA and protein sequences in the internal region altered by the mutagenic oligonucleotides 282-312A and 307-339S. The first line gives the codon numbers for *merA* or *merApe9*. The second line shows the MerApe9 protein sequence. The third line gives the synthetic sequence incorporated into *merApe9*. The fourth line shows the G+C-rich nucleotides found in wild-type *merA* that have been mutagenized in *merApe9*. The G+C composition in the mutagenized region (codons 387–336) has been lowered from 65% to 47%, and codon usage has been substantially altered to favor those codons found in highly expressed plant (30) and *E. coli* (31) genes.

was measured on a Jerome 431 mercury vapor analyzer (Arizona Instrument, Tempe) in the following modification of the manufacturer's recommended procedures (18). Approximately 5–10 seedlings (10–14 day old, 10–25 mg total wet weight) were incubated in 2 ml of assay medium (50 mM Tris·HCl, pH 6.8/50 mM NaCl/25 μM $HgCl_2$) in a 16 × 130 mm test tube with a side arm for gas removal. The $HgCl_2$ was added separately to initiate the assay. The amount of Hg^0 produced was assayed by bubbling air through the bottom of sample for 12 sec at 3 cm^3/sec and measuring the released Hg^0. The time zero assay was taken immediately after the seedlings were placed in the medium. The sample was then reassayed every minute for 10 min. The volatilized Hg^0 was measured by passing the air sample released from the side arm directly over the gold foil membrane resistor of a Jerome 431 mercury vapor analyzer. The instrument was repeatedly standardized with known quantities of Hg^0 (10–200 ng), reduced from $HgCl_2$ with excess $SnCl_2$. The amount of mercury evolved was normalized by dividing the number of nanograms of Hg^0 measured by the number of milligrams of seedling tissue in the assay.

Steady-State RNA Levels. Total RNA was prepared from transgenic and control plants (37). This RNA was resolved by electrophoresis on 1% agarose/formaldehyde gels and blotted to a nylon membrane (38). The membrane was probed with *merApe9* amplified by PCR from pNS2 with the 5′S and 3′A oligonucleotides and ^{32}P-labeled by a random primer method to a specific activity of 1–2 × 10^8 cpm/μg. Filters were stripped and reprobed with a clone encoding the soybean 18S rRNA gene (39). The levels of *merA* mRNA and 18S rRNA probe bound to the appropriate bands on the filters were quantified on a Molecular Dynamics PhosphorImager. The relative levels of *merA* mRNA in the various samples were obtained by first normalizing to 18S rRNA levels and then dividing all values by the highest mRNA level.

RESULTS

Initial attempts in our laboratory to express the bacterial *merA* gene from Tn*21* in transgenic plants and to produce Hg^{2+}-resistant plants were unsuccessful (18) in spite of the use of very efficient plant expression systems. No *merA*-encoded protein or full-length *merA* RNA was detected. The original 1695-nt *merA* coding sequence in Tn*21* is G+C rich (≈67%), contains 218 CpG dinucleotides (40), and is skewed toward GpC-rich codons, which are uncommon in plant (30) and *E. coli* genes (31). The A+T-rich *Bacillus thuringiensis* toxin coding sequence is also poorly expressed in transgenic plants, but substantial increases in protein expression were obtained with modifications that increased the G+C nucleotide composition of only 3% of the codons (41). Therefore, we constructed a modified *merA* gene, *merApe9*, using an OE-PCR strategy, which is shown in Fig. 1*A*. Codons 287–336 (i.e., 9% of the coding region) were replaced with nucleotide combinations and codons more common to highly expressed plant and *E. coli* genes (Fig. 1*B*), and 15 bp of the 5′ region immediately upstream from the initiator ATG codon was replaced with consensus plant and *E. coli* translation signals. The full-length OE-PCR product was ligated into an *E. coli* expression vector under control of the *E. coli lac* promoter. The ligation mixture was transformed into a "wild-type" *E. coli* strain, SK1592, containing the pPB111-47 plasmid, which encodes the *mer* operon, has mercury transport functions, but has a disruption in the *merA* gene. Ampicillin-resistant transformants were selected and replica plated onto media containing 100 and 200 μM Hg^{2+}. One colony that grew exceptionally well on 200 μM Hg^{2+} was characterized further and contained *merApe9* in plasmid pNS2 (Table 1 and *Materials and Methods*).

The *merApe9* construct was first assayed for reductase activity in *E. coli*. The size of the zone of sensitivity around disks containing mercuric ion are shown for various strains in Table 1. The parent *E. coli* strain, SK1592/pPB111-47 (42), was supersensitive to mercury, producing a large ring of growth inhibition (Hg^{SS}; 30 mm). This strain has the mercury transport system and pumps even very low levels of ionic mercury into the cell, but cannot reduce Hg^{2+} to the nontoxic Hg^0. When the supersensitive strain also contained the pNS2 plasmid, it showed full levels of Hg^{2+} resistance (SK1592/pPB111-47/pNS2; 14 mm). pDU202 has an intact *merA* gene and is the parent plasmid to pPB111-47. pNS2 alone increased slightly the resistance of wild-type *E. coli* strain SK1592 to Hg^{2+}. This confirmed a recent report that R plasmids mutated in the transport functions *merT* and *merP* produce weak mercury resistance (26). These results demonstrated that *merApe9* encoded a fully functional mercuric ion reductase. The sequence of the *merApe9* gene in pNS2 contained a few unintended mutations, which were evidently introduced during the OE-PCR protocol. These changes produced two conservative amino acid changes in the MerApe9 protein (underlined in Fig. 1*B*; see *Materials and Methods*).

Based on reports that *E. coli* strains with the intact *mer* operon reduced ionic gold, Au^{3+} (17), we tested the strain expressing *merA* alone and found it was substantially resistant to Au^{3+} relative to the control strain (Table 1). Strains with the intact *mer* operon were no more resistant to Au^{3+}, suggesting that the transport functions were not important to Au^{3+} resistance.

The *merApe9* sequence from pNS2 was subcloned into a plant expression vector, which placed *merApe9* under control of the constitutive plant cauliflower mosaic virus 35S promoter and plant nopaline synthase 3′ polyadenylylation signals in a T-DNA binary vector. This construct was transformed into *Arabidopsis* (RLD ecotype) embryos from an *A. tumefaciens* bacterial host (see *Materials and Methods*). *Arabidopsis* shoots containing the *merApe9* sequence were regenerated (T0 generation), selecting for kanamycin resistance encoded on the plasmid vector, and allowed to set seed (T1 generation).

Second, T2, and later generation seeds, seedlings, and plants from several independent *merApe9* transgenic lines were resistant to 50–100 μM $HgCl_2$ in the growth medium. The results for segregants of transgenic line no. 1 are shown in Fig. 2*A*. Typically 50–100% of the seeds from most *merApe9* lines germinated at these concentrations. On mercury-containing agar, they had normal roots and leaves of darker than normal green color. Seeds, seedlings, or mature plants from nontransgenic controls (RLD; Fig. 2*A*), or Mendelian segregants lacking the transgene (data not shown), and transgenic lines containing other T-DNA constructs (*35S/GUS* and *ACT7/GUS*) would not germinate and/or died shortly after germination on 25 μM or greater concentrations of $HgCl_2$. At toxic

Table 1. Filter disk assay for metal ion sensitivity in *E. coli*

Strain and plasmid(s)	Phenotype on Hg	Size of zone of sensitivity, mm: $HgCl_2$, 100 μM	Size of zone of sensitivity, mm: $HAuCl_4$, 200 μM
SK1592 pBSSKII	S	24	20
SK1592/pNS2	R	21	16
SK1592/pPB111-47/pBSSKII	SS	30	20
SK1592/pPB111-47/pNS2	R	14	15.5
SK1592 DU1040 pDU202 pBSSKII	R	15	16

Each 6-mm-diameter disk contained 2 μl of freshly prepared metal salt stock solution at the concentration indicated. The values given are the average of two replicates. There was <1 mm difference for any value in the two experiments. S, sensitive; SS, supersensitive; R, resistant.

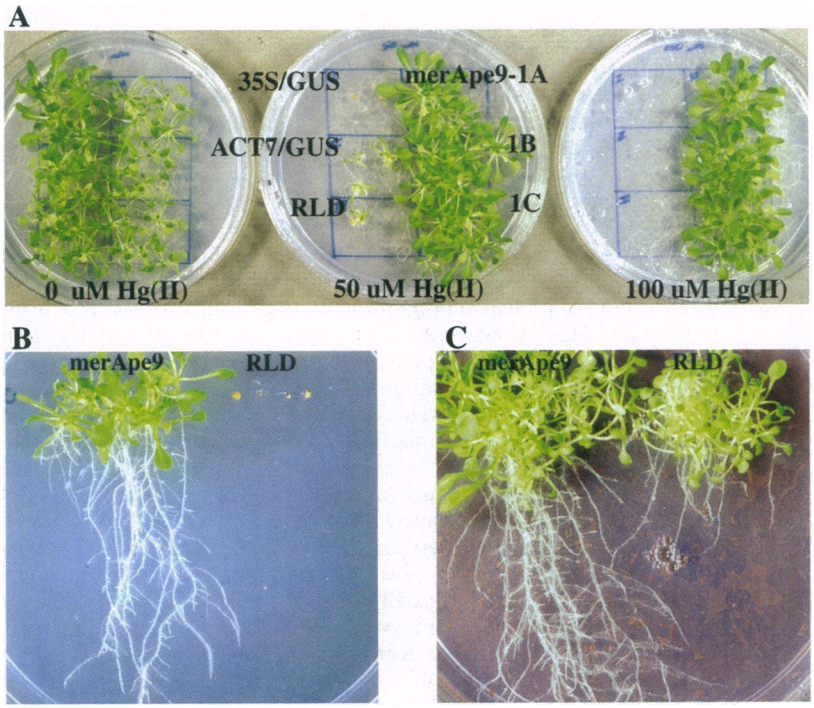

FIG. 2. Transgenic *Arabidopsis* containing the *merApe9* construct were resistant to mercury and gold ions. (*A*) Seeds from *Arabidopsis* transgenic plant line no. 1 (T3 segregating populations 1A, 1B, and 1C) expressing the *merApe9* gene (right-hand three sectors on each plate) germinated and grew on plant growth medium containing 0, 50, and 100 μM $HgCl_2$. Transgenic controls expressing unrelated genes (*35S/GUS* and *ACT7/GUS*) and nontransgenic RLD control seeds (left-hand three sectors of each plate) seldom germinated and if so subsequently died on medium with 50 or 100 μM $HgCl_2$. Some of the most highly resistant plant lines including line no. 1 do not grow as well on medium lacking $HgCl_2$ (left hand plate) and appear to require $HgCl_2$ for normal growth (see *Discussion*). (*B*) Seeds from a *merApe9*-expressing line (1A) and RLD controls were germinated on 50 μM $HgCl_2$ medium (as above), and the plates were incubated vertically to get a better view of the developing root systems. (*C*) Seeds from a *merApe9*-expressing line (1A) and RLD controls were germinated on medium containing toxic levels of gold ions (150 μM $HAuCl_3$).

concentrations of $HgCl_2$ that killed all RLD seeds, seedlings, and juvenile plants (data not shown), the transgenic *merApe9*-expressing lines showed vigorous root and shoot growth (Fig. 2*B*). Seven of the nine independent transgenic lines were resistant to 25–100 μM mercury in the medium, although one of these seven resistant lines, no. 4, grew very slowly for the first few weeks on mercury and then grew normally. A few of the most resistant lines, nos. 1 (Fig. 2*A*), 2, and 6, grew more poorly than wild-type controls on mercury-free medium, but grew as well on media containing 25–100 μM mercury as unchallenged control plants. Some potential causes of this apparent mercury requirement are discussed below. Transgenic lines were also more resistant to gold ion, Au^{3+}, than wild-type strains, which showed a significant reduction of root growth at sublethal concentrations of $HAuCl_4$ in the medium (Fig. 2*C*).

The level of mercuric ion reduction was measured in intact plants. Transgenic *merApe9*-containing *Arabidopsis* seedlings or small plants were suspended in liquid medium, 5 μM Hg^{2+} was added, and air was bubbled through the sample and into a mercury vapor analyzer as shown in Fig. 3*A* and described in *Materials and Methods*. The results for segregants from transgenic line no. 1 are shown in Fig. 3*B*. Each of the segrating lines gave approximately the same rate of mercury evolution, demonstrating that the mercury vapor assays were quite reproducible. Transgenic *merApe9*-containing seedlings derived from most of the independent lines reduced Hg^{2+} to volatile Hg^0 severalfold more efficiently than control seedlings. The same was true for young plantlets (data not shown). Sublethal mercury concentrations were used so as not to kill the control plants, but higher mercury concentrations gave similar results for transgenic plants. The rapid evolution of Hg^0 by transgenic seedlings and plants relative to controls confirmed that resistance was due to increased reduction of Hg^{2+} by the MerA enzyme.

Total RNA was prepared from several independent transgenic lines containing *merApe9*, plant segregant lines derived from these lines that had lost *merApe9*, and control plants. The RNA was resolved by formaldehyde/agarose gel electrophoresis (see *Materials and Methods*), blotted to a nylon membrane,

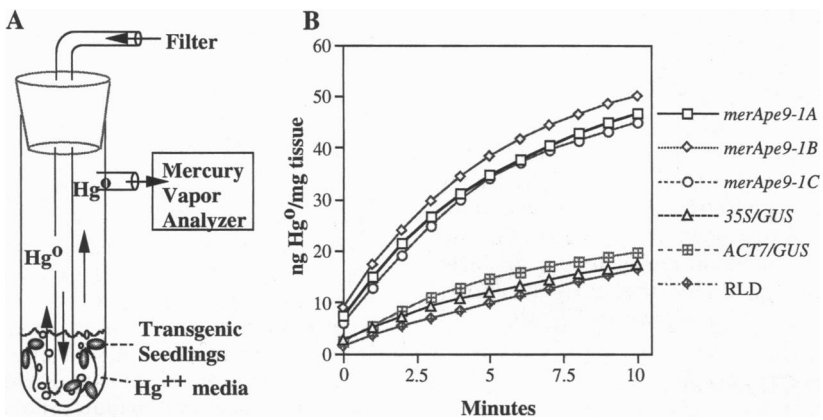

FIG. 3. Transgenic plants with the *merApe9* construct volatilized Hg^0 and therefore express MerA protein. (*A*) Seedlings were placed in a small sidearm test tube in assay medium. Hg^{2+} was added to start the reaction, air was bubbled through the tube, and the level of volatilized Hg^0 was measured in a Jerome mercury vapor analyzer (see *Materials and Methods*). (*B*) Transgenic seedlings expressing the *merApe9* gene (1A, 1B, 1C) catalyzed significant reduction of Hg^{2+} to Hg^0 relative to the background of chemical reduction seen in control RLD plants or transgenic plants expressing other genes (*35S/GUS* and *ACT7/GUS*). For each of the three *merApe9*-expressing transgenic samples shown above, ≈10 mg of seedlings evolved ≈500 ng of Hg^0 during the 10-min assay period (see *Materials and Methods*).

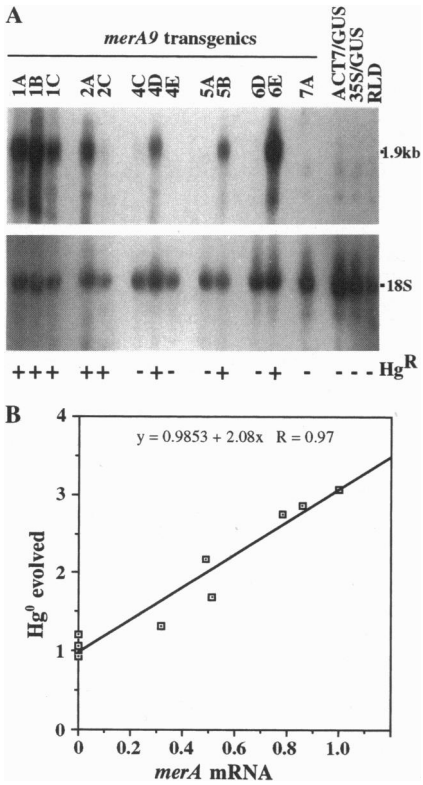

FIG. 4. Correlation of *merApe9* RNA expression levels and MerApe9 reductase activity in plants. (*A*) RNA samples from various transgenic lines were resolved on agarose/formaldehyde gels, transferred to a nylon membrane, and probed with merApe9 (*Upper*) and 18S rDNA (*Lower*). The autoradiograms were exposed to x-ray film for 3 days and 30 min, respectively. Hg^R, mercury resistance. (*B*) The levels of *merApe9* RNA in *A* quantified on a PhosphorImager and normalized to 18S rRNA levels are plotted against the relative levels of Hg^0 evolved by the individual transgenic lines in a mercury vapor evolution assay as shown for line no. 1 in Fig. 3. Plant line 6E had the highest level of *merApe9* mRNA, and all mRNA levels were normalized to this value.

and assayed for the levels of *merApe9* mRNA. As shown in Fig. 4*A*, the levels of *merApe9* mRNA varied among the transgenic lines examined (e.g., nos. 1, 2, 4, 5, 6, and 7), suggesting that the position of T-DNA insertion or copy number in the host plant genome affected expression of the transgene. Some segregant lines (e.g., 2C, 4C, 4D, 5A, and 7A) apparently did not make *merApe9* mRNA or made much lower amounts. Those lines expressing high levels of *merApe9* RNA were mercury resistant (Hg^R at the bottom of Fig. 4*A*).

The level of *merApe9* mRNA in each 1.9-kb band in Fig. 4*A* was then normalized to the level of 18S rRNA using a PhosphorImager to quantify the levels of isotope bound to the Northern filter (see *Materials and Methods*). Each of these lines and segregants was also assayed for the level of Hg^0 evolution per mg of seedling tissue as described above in Fig. 3*B*. The level of *merApe9* mRNA expression correlated surprisingly well and in a linear relationship with the level of Hg^0 evolution (correlation coefficient, $r = 0.97$) as shown in Fig. 4*B*.

DISCUSSION

Toxic metal ion pollution is perhaps one of our most difficult environmental problems. Unlike organic and even halogenated organic pollutants, which can be degraded in the soil, metals are essentially nonmutable. The electrolytic, chemical leaching, and *in situ* immobilization technologies for cleaning contaminated sites are all quite expensive, particularly in light of how large some of these sites are (43). With the exception of approaches like vitrification, most *in situ* metal ion remediation schemes require some mechanism for increased mobilization of the metal ion. This raises the possibility of further endangering local wildlife or adjacent ecosystems not already affected. Although a full examination of the impact on the ecological system surrounding a contaminated site would be required, we believe that metal ion-reducing plants may offer a safe and cost-effective means of remediation.

As a case study, we will consider a mercuric ion reduction strategy for remediation of a site contaminated with Hg^{2+} by industrial bleaching operations or past agricultural practices. The Hg^{2+} slowly leaches from the site into the water system and concentrates in plant, insect, fish, and, ultimately, bird and mammalian populations. The mercury is subjected to the standard bio- geo-chemical cycle for mercury (44), and most of it is bound to thio-organics and humic substances in the soil. Bacteria expressing the *mer* operon are ubiquitous to these sites, and they reduce and continually volatilize elemental mercury (44). However, plants generally control most of the energy in these ecosystems (45, 46), and plants often have an excess of reducing power from photosystem I, which we propose can be tapped to reduce metal ions. A selection of appropriate transgenic MerA-expressing plants for each particular habitat could accelerate the biological transformation of bound Hg^{2+} to Hg^0 and its loss from these sites, over and above what is already processed by bacteria. The placement of these improved plants around pollution sources and at their points of discharge and collection could prevent toxic Hg^{2+} accumulation at and transport from these locations. This remediation strategy could be used to divert mercury away from our most sensitive wetland areas and away from local animal populations. Once plants have transformed the mercury, air movement will dilute it to nontoxic levels and remove the mercury from these areas (47). This mercury will be reoxidized in the atmosphere and return diluted to terrestrial and marine sediments bound to sulfur and carbon compounds (44). Ultimately, this should lead to a more natural distribution of mercury in the environment and lower mercury concentrations to nontoxic levels in those areas where it threatens wildlife and human populations. Nonetheless, for this to be an environmentally sound, long-term global strategy for removing mercury from a site, only low levels of mercury can be introduced into the environment from new anthropomorphic sources. At present, high concentrations of toxic mercury continue to accumulate in some locations.

The original goal of our research was to explore metal ion reduction as a general approach to metal ion resistance and metal sequestration in plants. Although metal ion reduction is not recognized as commonplace for plants, it has recently been suggested that plants can reduce toxic Cr(VI) to less toxic Cr(III) (48). Our results suggest that at a minimum mercury reduction can be engineered into plants and most likely used as a selectable genetic marker in the laboratory. What about reduction strategies for resistance to and processing of other toxic metal ions? Like mercuric ion, some other metal ion pollutants such as Cu^{2+} and Pb^{2+} have a strong tendency to be reduced to the metallic state. Unlike volatile mercury, metallic copper and lead should precipitate in the cell, and, thus, most scenarios for remediation of these metals by reduction would involve removal of the metal-containing plants from the site. It may be possible to find bacterial reductases for these and other divalent metal ions or to engineer this capability into MerA using accelerated mutagenesis protocols. Previous work (17) and our initial data suggest that Au^{3+} may already be a substrate for MerA-catalyzed reduction. Mechanistic studies have demonstrated that MerA already binds Ag^+, Cu^{2+}, and Cd^{2+} (49) as competitive inhibitors (e.g., K_i = 1, 14, and 18 μM, respectively) about as well as it binds Hg^{2+} (K_m = 12 μM). Like Hg^{2+}, these metals form strong thiol salts. Thus, only

minor changes in sequence around the active site may be required to gain reduction capacity for these and other toxic metals.

Several of the most mercury-resistant *merApe9*-expressing lines with high *merApe9* mRNA levels (e.g., 1A, 2A, 5B, and 6E) grew better on mercury-containing medium than on control medium (compare the left and right plates of Fig. 2*A*). These lines required some mercury in the medium for optimum growth. While further research will be required to dissect this interesting result, we propose that expression of *merApe9* made the plants deficient in some essential metal ion or organic metabolite due to reduction of that compound. MerA is reported to use at least one thioorganic [5,5-dithiobis(2-nitrobenzoate)] as a substrate (49). Following this hypothesis further, in the presence of mercury, the enzyme would be occupied with $RSHg^+$ (see Introduction) for which it has a greater affinity, inhibiting the nonspecific enzyme activity toward the essential compound. This trait could work to the advantage of any phytoremediation strategy. If mercury-requiring transgenic plant lines did not compete well in areas that were not mercury contaminated, it would offer some level of containment of the transgenic plants within mercury polluted sites.

The data on mercuric ion reduction and resistance presented herein suggest that there are potentially viable molecular genetic approaches to the phytoremediation of metal ion pollution. We plan to determine if *merA* expression confers efficient mercuric ion reduction and resistance to distantly related plant species and consider their use in various remediation strategies. The relative contributions of the altered flanking sequences, optimized codon usage, and decreased percentage of G+C to the efficient expression of *merA* and Hg^{2+} resistance remains to be examined. While one might imagine that the changes made to *merApe9* gene structure increased transcription rates, stabilized the mRNA, and/or improved translational efficiency, significant future effort will be required to understand the mechanisms resulting in the efficient expression of *merApe9*.

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