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Transgenic Tobacco Plant Expressing Environmental *E. coli merA* Gene for Enhanced Volatilization of Ionic Mercury

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The practicability of transgenic tobacco engineered to express bacterial native mercuric reductase (MerA), responsible for the transport of Hg^{2+} ions into the cell and their reduction to elemental mercury (Hg^0), without any codon modification, for phytoremediation of mercury pollution was evaluated. Transgenic tobacco plants reduce mercury ions to the metallic form; take up metallic mercury through their roots; and evolve the less toxic elemental mercury. Transformed tobacco produced a large amount of *merA* protein in leaves and showed a relatively higher resistance phenotype to $HgCl_2$ than wild type. Results suggest that the integrated *merA* gene, encoding mercuric reductase, a key enzyme of the bacterial *mer* operon, was stably integrated into the tobacco genome and translated to active MerA, which catalyzes the bioconversion of toxic Hg^{2+} to the least toxic elemental Hg^0, and suggest that MerA is capable of reducing the Hg^{2+}, probably *via* NADPH as an electron donor. The transgenic tobacco expressing *merA* volatilized significantly more mercury than wild-type plants. This is first time we are reporting the expression of a bacterial native *merA* gene *via* the nuclear genome of *Nicotiana tabacum*, and enhanced mercury volatilization from tobacco transgenics. The study clearly indicates that transgenic tobacco plants are reasonable candidates for the remediation of mercury-contaminated areas.

Keywords: Bioaccumulation, mercuric reductase, *merA*, transgenic, phytoremediation

Mercury is among the most perilous of the heavy metals, primarily because its charged species have great affinity for the thiol group on cysteine residues of proteins and other important biological molecules [5]; this makes mercury a potent neurotoxin and one of the most detrimental and toxic environmental pollutants. Even an extremely low level of exposure to mercury can cause permanent damage to the human central nervous system. However, mercury levels have risen owing to environmental contamination from human activities, such as the burning of coal and petroleum products, and the use of mercurial fungicides in paper making and agriculture. Using mercury catalysts in industry resulted in a consequent release of mercury into air, water, and land. These activities can increase local mercury levels several thousand folds above background levels. Microorganisms in contaminated environments have developed resistance to mercury and are playing a major role in natural decontamination. The most important detoxification mechanism is the enzymatic reduction [16, 27] of Hg^{2+} (toxic form) to Hg^0 (metallic and least reactive form). The biotransformation is mediated by mercury reductase, an inducible NADPH-dependent flavin-containing disulfide oxidoreductase enzyme. This enzyme is encoded by plasmid-borne *merA* gene, an integral part of the mercury-resistant *mer* operon [27]. These resistance mechanisms are often encoded by plasmids [4, 8, 26] or transposons [15] in the bacterial genome. Current methods to clean up heavy-metal-contaminated soils are quite expensive, environmentally invasive, and labor intensive, and thus many mercury-polluted areas are presently left unreclaimed. An alternative cost-effective approach is phytoremediation, which is the use of plants to clean up contaminated environments. Several studies have successfully integrated bacterial *mer* genes into plant genomes to create superior plants for phytoremediation of mercurial-contaminated sites, based on the *merA*-mediated mercury reduction and volatilization mechanism [3, 21, 23, 24]. Converting tobacco to a mercury volatilizing plant could create a multiple-use crop that can be grown in mercury-contaminated soil.

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Since tobacco is much larger than the model *Arabidopsis* plant, and has extensive root systems capable of extracting mercury, its potential for mercury evaporation is greater. Thus, the goal of the present study has been towards the development of mercury-resistant *Nicotiana tabacum* transgenic plants to remove inorganic mercury from contaminated sites by expressing the bacterial native *merA* gene *via* the nuclear genome of tobacco, without any codon modification.

Materials and Methods

Collection of Water Samples, Bacterial Screening, and Transformation of Plasmid

Water samples were collected from different aquatic environments representing distinct geographical regions of India; namely, site-I (YR-I) and site-II (YR-II) of Yamuna River (YR), Delhi; Kalu River (KR), Bombay; YR near Guru Tegh Bahadur Hospital (GTB), Delhi; Hindon River (HR), Ghaziabad; Kalindi Kunj (KK), Delhi; and Hoogly River (H_gR), Kolkata. An 8th sample collected from Dal Lake (a pristine-type lake), Srinagar, Kashmir was considered as the control. The initial screenings of *E. coli* were performed on eosin methyl blue agar plates. The selected strains were subjected to differential and selective growth media, followed by various biochemical studies for their identification. $HgCl_2$ sensitivity of the strains was also tested by determining their minimum inhibitory concentration (MIC) levels at which no bacterial growth was observed. Plasmid DNA was isolated by the method of Birnboim and Doly [2] for all the samples, and the location of the *mer* operon was determined by transforming the isolated plasmids into host DH5α cells as described by Hanahan [10]. Plasmids from transformed colonies were compared with the plasmid profile of the wild-type strains.

PCR Amplification of *merA* and Its Cloning into pGEM-T and pBI121

The complete ORF of *merA* was amplified using the gene-specific N-terminus forward primer (5' CGG GAT CCA TGA GCA CTC TCA AAA TCA CC 3') and C-terminus reverse primer (5' TCC CCC GGG ATC GCA CAC CTC CTT GTC CTC 3'), using plasmid DNA as the template. The reaction mixture contained PCR buffer [100 mM Tris (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin], 0.2 mM dNTPs, 50 pmol/μl each of forward and reverse primers, 1 unit of *Taq* DNA polymerase, and 50 ng of plasmid DNA. The PCR conditions included denaturation at 95°C for 1 min, primer annealing at 63°C for 2 min, and extension at 72°C for 3 min, followed by an initial denaturation at 95°C for 5 min, and final extension at 72°C for 5 min for 30 cycles in a 50-μl reaction volume. Amplicons from the YR-II and HR samples were gel purified with a Gene*i*Spin Gel extraction kit (Gene*i*), and cloned into the pGEM-T Easy vector (3,015 bp; Promega) following the manufacturer's protocol. The underlined hexamer sequences in the N-terminus forward primer and in the C-terminus reverse primer indicate the *Bam*HI and *Sma*I restriction sites, respectively; those were placed in the primers to facilitate subsequent cloning of the amplified full-length *merA* into the pBI121 plant expression vector (13 kb; Clontech). Blunt end ligation followed by cohesive end ligation was performed, following the manufacturer's protocol (recombinant pBI121-*merA* construct map; Fig. 1). The ligated product of pBI121-*merA* was further transformed into competent *E. coli* DH5α cells.

Mobilization of pBI121-*merA* Construct into *Agrobacterium tumefaciens* GV3101

The recombinant pBI121-*merA* construct was mobilized into *A. tumefaciens* GV3101 by the freeze-thaw method [13], with minor modifications. For this preparation, *A. tumefaciens* GV3101 was grown in 50 ml of YEM medium (0.04% yeast extract, 1% mannitol, 0.01% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.05% K_2HPO_4) supplemented with 25 mg/ml rifampicin and 10 mg/ml gentamycin at 28°C with vigorous shaking until its OD_{600} reached to 0.5–0.8. The culture was chilled on ice and centrifuged at 5,000 rpm for 5 min at 4°C to pellet down the cells. The pellet was resuspended in 1 ml of 20 mM $CaCl_2$, and 100 μl of resuspended competent cells were aliquoted in pre-chilled microfuge tubes and stored at −80°C for further transformation purposes. For *A. tumefaciens* GV3101 transformation, ~3 μg (~300 μl from stock of 10 ng/μl) of ligated pBI121 vector DNA (recombinant pBI121-*merA* construct) was added into Eppendorf tubes containing frozen competent cells of *Agrobacterium*, and cells were thawed in a 37°C water bath for 5–6 min. Afterwards, 1 ml of

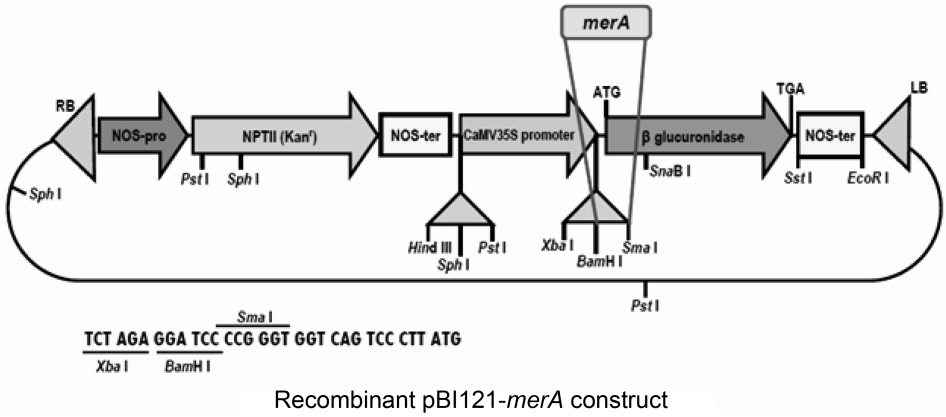

Fig. 1. Recombinant pBI121-*merA* construct map.
This diagram shows the integration of the environmental *E. coli* native *merA* gene into the plant expression vector pBI121 (13 kb) at *Bam*HI and *Sma*I restriction sites under control of the CaMV35S promoter.

YEM medium was added to the same tube and incubated at 28°C for 2–4 h with gentle shaking. The grown culture was poured (~200 µl) on YEM agar plates with 50 mg/ml of kanamycin for the selection of transformed colonies. The cultured plates with antibiotics were kept at 28°C for 2–3 days. The colonies of transformed *A. tumefaciens* GV3101 appeared after incubation of 36–42 h. The colonies of transformed *Agrobacterium* were checked by *merA* PCR and restriction digestion analyses.

Development of *Nicotiana tabacum* Transgenic Plants

Two-week-old leaf-derived calli from *Nicotiana tabacum* cv. Xanthium of size 0.2–0.4 mm were prepared and cocultured with YEM (supplemented with 50 µM acetosyringone)-grown bacteria following the standard procedure. After several washes with cefotaxime, calli were placed on MS basal medium, and 4–5 days later calli were transferred to the same medium with kanamycin (35 mg/l). After 4 weeks, plants were transferred to MS medium supplemented with kanamycin (35 mg/l), NAA (0.5 mg/l), and kinetin (1.0 mg/l). Regenerated plants were transferred to jars containing 1/2 basal MS media for further growth and development. After 4 weeks, plants were removed from the jars and transferred to pots containing soilrite.

Molecular Characterization of Transgenic Plants

Genomic DNA was isolated from plant leaves following the method of Edward *et al*. [7], and *merA* PCR was performed following the conditions similar to the bacterial *merA* amplification. For Southern blot, 2–3 µg of plant DNA was digested with *Sma*I and *Bam*HI restriction enzymes by incubating at 25°C for 12–16 h and 37°C for 2 h, respectively. Restricted DNA was transferred onto a Hybond N^+ Nylon membrane; probe labeling and detection were performed with the help of an ECL direct nucleic acid labeling and detection system from Amersham Pharmacia. Full-length amplified *merA* from the recombinant pBI121-*merA* construct was used as a probe.

Transgene Expression Analysis

Total plant RNA was isolated for reverse transcriptase (RT)–PCR by using an RNeasy plant minikit (Qiagen) following the manufacturer's protocol. The RT–PCR was performed by converting 1 µg of RNA into cDNA by using the AMV reverse transcriptase of the One-step RT–PCR kit (Qiagen), and further amplification was done by using *merA*-specific primers. RT–PCR conditions were RT at 50°C for 30 min, initial activation at 95°C for 15 min, denaturation at 95°C for 1 min, annealing at 63°C for 2 min, and extension at 72°C for 3 min, run for 30 cycles, and final extension was performed at 72°C for 7 min. For the Northern blot, total plant RNA was isolated by the conventional GTC (acid-guanidinium thiocyanate) method and resolved on 1% agarose gel containing ethidium bromide, 1× MOPS (sodium acetate, EDTA, and MOPS), and 6% formaldehyde. Bands were transferred to a Hybond N^+ nylon membrane. Full-length amplified *merA* from recombinant the pBI121-*merA* construct was used as a probe. Probe labeling and detection were performed with GE Healthcare's Amersham Gene Images AlkPhos direct labeling and detection system (CDP-*Star*).

Cloning and Expression of Mercuric Reductase Gene in pQE-30UA

Amplified *merA* from YR-II was gel purified and ligated into the bacterial expression vector pQE-30UA (Qiagen), as described by the manufacturer's instruction, and then transformed into competent *E. coli* BL21(DE3) pLysS cells (Novagen).

Standardization of Optimal Expression Conditions for *merA* Protein

Transformed *E. coli* BL21(DE3) pLysS cells with pQE-30UA-*merA* (termed as QIA*express* cells from Qiagen) were grown in Luria broth (LB) and induced with 1.0 mM IPTG. Pellet was collected at different time intervals and checked for protein expression on 12% SDS–PAGE and stained with Coomassie brilliant blue-R250. Different IPTG concentrations (0.5 mM–1.5 mM) and different temperature combinations (28–37°C) were used to standardize for optimal expression.

Overexpression, 6×His-*merA* Affinity Purification, and Anti-*merA* Polyclonal Antibody Generation

Fusion protein was purified under denaturating condition with the 6×His-tag of the QIA*express* system using immobilized metal-chelate affinity chromatography (IMAC). For overexpression of MerA, QIA*express* cells were grown in LB medium amended with 50 µg/ml ampicillin. Fractions of 1.0 mM IPTG-induced culture were harvested at different time intervals and loaded on a Ni-NTA resin column, and the desired protein was eluted with buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing different concentrations of imidazole (60, 80, 100, 150, 200, and 250 mM). Elutants along with crude extract were checked on 12% SDS–PAGE as described by Laemmli [17], and fractions containing the desirable protein were subjected to dialysis. Mercuric reductase purified from the 6×His conjugate was used as an antigen for immunization of rabbit using complete/incomplete Freund's adjuvant (Difco Laboratories). ELISA was performed for all the collected serum samples to check the antibody titer, using goat anti-rabbit IgG-HRP, and absorbance was read at 492 nm with 620 nm as the reference filter in a iMark Microplate Reader (Bio-Rad).

Western Blot Analysis for *merA* Protein Expression

Total plant cell protein was extracted, quantified, and resolved on 10% SDS–PAGE. The gel was transferred to a Hybond-C extra nitrocellulose membrane, followed by Ponceau-S staining, and a Western blot was performed according to the manufacturer's protocol for the Super Signal West Pico Chemiluminescent Substrate Kit from Pierce Biotechnology, U.S.A., using goat anti-rabbit IgG secondary antibody.

Inorganic Mercury Response and Mercury Volatilization Assay for Transgenic Lines

Following 3 months of maintenance upon MS medium, all the PCR and RT–PCR-selected *merA* transgenic tobacco lines along with wild-type control plants were subjected to increasing concentrations of $HgCl_2$ (20, 40, 60, 80, 100, 120, 140, and 160 µM). The individual plantlets (transformed and wild type) were evaluated for growth and survival after 2 weeks. In another experiment, following the initial maintenance on MS medium for 3 months, transformed and untransformed wild plants were transferred to soilrite pots. After 2 weeks of growth, all the plants were transferred to sterile soilrite pots amended with the increasing concentrations of mercury mentioned as above and evaluated for their survival after 2 weeks.

A mercury vapor analyzer (Jerome 431-X, Arizona Instruments) was used to evaluate the volatilized mercury released from the plant tissues. Each tissue culture (3 months old) and soilrite-grown plant (2 weeks old) samples were rinsed free of medium/soil, and dissected into root, shoot, and stem fractions. The plant samples (2.0 g) were incubated in 300 ml of volatilization medium containing 25 µM $HgCl_2$ in a 2.0-l flask with a side arm for gas control. Different plant

organs were suspended in fresh $HgCl_2$ solution and the mercury vapor evolved was sampled by sparge evacuation of the headspace over the 1-week volatilization assay. All the Hg volatilization assays were performed in triplicate.

RESULTS

Bacterial Screening, Plasmid Isolation, and Transformation

The collected water samples had varying physiochemical properties for pH, temperature, and turbidity. The maximum mercury load was found in the Yamuna River (YR) sample as compared with the other water samples. The main reason for this difference may be that most of the mercury-containing industrial effluents present in Delhi enter into the YR. The YR-II sample had a mercury content ~3.76 ppm, which is nearly 3 times higher than the prescribed limit (1.0 μg/l) of the WHO. All isolated strains showed the presence of ~24-kb size plasmid [9]. Transformation of plasmids isolated from Hg^r *E. coli* isolates into mercury-sensitive (Hg^s) *E. coli* DH5α cells yielded transformants in each case on LB agar plates supplemented with different concentrations of $HgCl_2$ to which the donor strains were resistant.

Amplification, Cloning, and Sequence Analysis of Putative *merA*

Full-length *merA* was amplified from 6 *E. coli* samples and a 1,695 bp putative *merA* was detected for all the samples. Owing to the enhanced resistance towards mercury through consistently increasing exposure to mercury compounds by industrial pollution, as reported earlier [1], amplified products from the YR-II sample were utilized for all the cloning and transgenic development studies. Amplicons of *merA* from the YR-II and HR samples were cloned into the pGEM-T Easy vector and sequenced. Sequencing results confirmed that the *merA* sequences of both samples were homologous to the *merA* sequence already available in the NCBI database (GenBank Accession No. NC_002134).

Cloning of *merA* into pBI121 and Transformation of *Agrobacterium* GV3101

Purified PCR products obtained from the YR-II sample were cloned successfully into the pBI121 plant expression vector at the *Sma*I and *Bam*HI sites, and the recombinant construct pBI121-*merA* was then mobilized into the *Agrobacterium* strain. The kanamycin-resistant colonies of *Agrobacterium* were screened by plasmid isolation and back transformation of the plasmid into *E. coli* DH5α cells, followed by *merA* amplification.

Development, Screening, and Molecular Analysis of Transgenics

A *merA* gene driven by the CaMV35S promoter was introduced into *Nicotiana tabacum* plants through *Agrobacterium*-mediated transformation using leaf-derived calli as explants.

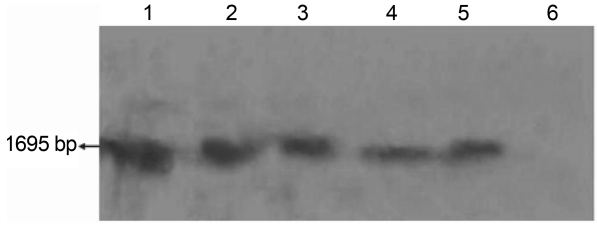

Fig. 2. Southern blot analysis.
Total DNA of transgenic lines and wild-type plant was digested respectively with *Bam*HI and *Sma*I, and hybridized with an ECL-based HRP-labeled *merA*-amplified insert fragment of pBI121. Lanes 1 to 5 show transgenic lines and lane 6 shows wild-type plant. All the five transgenic lines (T*Nt1*, T*Nt2*, T*Nt3*, T*Nt4*, and T*Nt5*) were shown to have a strong signal of *merA* integration into the tobacco genome.

Out of 127 putative plantlets, only 5 kanamycin-resistant lines (T*Nt1*, T*Nt2*, T*Nt3*, T*Nt4*, and T*Nt5*) were obtained by *merA* amplification. Integration of *merA* into the tobacco genome was also confirmed by Southern blotting (Fig. 2) and it was seen that, except for the wild-type plant, all strains had a strong signal. A 1,695-bp amplified fragment corresponding to the *merA* transcript confirmed the transcriptional-level expression of *merA* into transformed plants *via* RT–PCR. No amplification was noticed in RNA isolated from the wild-type plant. Northern blot (Fig. 3) revealed that the mRNA transcript for *merA* was not equally expressed; only 3 (T*Nt1*, T*Nt3*, and T*Nt4*) transgenic lines showed the high expression, whereas no observable expression was noticed for wild-type (W*Nt*) plants. Lines T*Nt2* and T*Nt5*, not as expected, showed a less observable level of *merA* expression.

Cloning of Putative *merA* into pQE-30UA and its Overexpression in Bacteria

Purified *merA* amplicons from the YR-II sample were cloned into the bacterial expression vector pQE-30UA and further

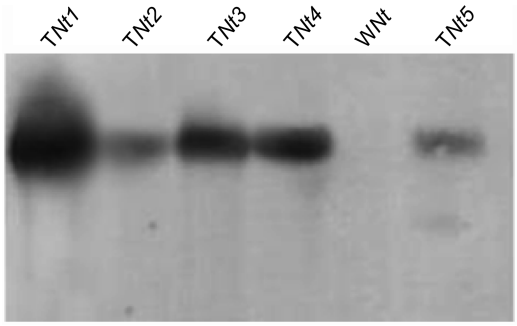

Fig. 3. Northern blot analysis of the wild type and transgenic lines.
Northern hybridization of transgenic plant lines of tobacco. Total RNA of all the lines were hybridized with the alkaline phosphatase (AlkPhos)-labeled *Bam*HI–*Sma*I *merA*-pBI121 fragment. T*Nt1*, T*Nt3*, and T*Nt4*, three of the transgenic lines, were shown to have a strong signal of transcriptional-level expression whereas wild-type (W*Nt*) tobacco indicated no signal expression.

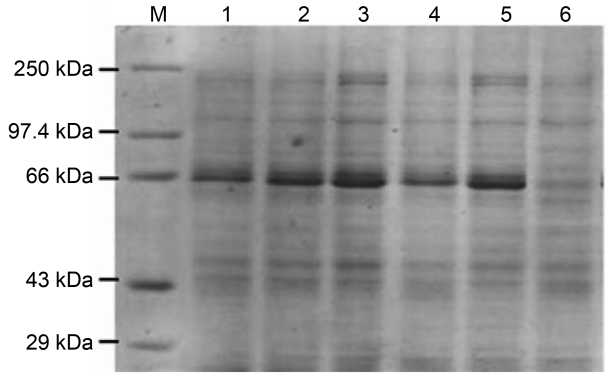

Fig. 4. SDS–PAGE analysis of pQE-30UA-*merA* containing *E. coli* BL-21(DE3) pLysS cells (QIA*express* cells) expressing the *merA* protein with IPTG induction.
Lane M shows the high-range protein marker of 29–205 kDa proteins; lane 6 shows the QIA*express* cells without induction (0 h) used as negative control; similarly, QIA*express* cells expressing *merA* protein with induction of 1 mM IPTG at 4, 8, 12, 16, and 20 h intervals are shown in lanes 4, 1, 2, 3, and 5, respectively.

transformed into *E. coli* BL21(DE3) pLysS cells. Clones were checked and conditions were standardized for optimal *merA* expression, and as evident from Fig. 4, the expression was increased with respect to time. MerA was purified with histidine tags and utilized as an antigen to raise polyclonal anti-*merA* antibody in rabbit. High antibody titers were found for blood collected at different time intervals from immunized rabbit.

Western Blotting

Transgenic lines T*Nt1*, T*Nt3*, and T*Nt4* overexpressed MerA, whereas less expression was observed in the T*Nt2* and T*Nt5* lines (Fig. 5). Proteins isolated from untransformed wild-type tobacco plants did not show any signal of MerA

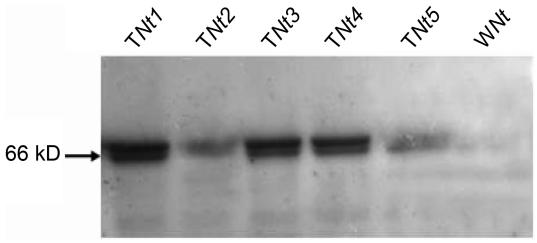

Fig. 5. Western blot analysis of overexpresser transgenic *merA* lines.
Western blot of total protein from transgenic *merA* lines T*Nt1*, T*Nt2*, T*Nt3*, T*Nt4*, T*Nt5*, and wild-type (W*Nt*) *Nicotiana tabacum* plants. Equal amounts of total protein were loaded on each lane, separated on a 10% SDS–PAGE gel, Ponceau-S stained, blotted on membrane, and probed with anti-*merA* polyclonal antibody raised in rabbit, anti-*merA*-labeled secondary antibody (goat anti-rabbit IgG) conjugated with horseradish peroxidase (HRP), and visualized with chemiluminescence. Purified *merA* protein (~66 kD) isolated from *E. coli* was included as a positive control (data not shown).

expression. The estimated *E. coli merA* protein size is 66 kDa, but in the case of plant that expressed MerA, the MW was slightly high (*i.e.*, ~69 kDa in all the transformed lines). This slight increase in *merA* protein MW may be due to glycosylation of proteins in plants cells.

Tolerance Towards Inorganic Mercury in Transgenic Lines

In tissue culture conditions, most shoots were fully able to resist up to 120 μM of $HgCl_2$ and 3 shoots were fully able to grow and resist the inorganic mercury up to 140 μM of $HgCl_2$ (Fig. 6, showing 2 shoots growing in Hg-amended media), whereas wild type was not able to tolerate more than 20–30 μM of $HgCl_2$. Transgenics T*Nt1*, T*Nt3*, and T*Nt4* in pots of soilrite, resisted up to 140 μM of $HgCl_2$, but T*Nt2* and T*Nt5* were not able to resist more than 80 and 100 μM of mercury, whereas untransformed wild-type plants did not survive beyond 20 μM mercury (data not shown). It was also seen that the wild-type untransformed control plants grew more vigorously than transgenic plants in soilrite pots with no mercury, which indicted that *merA* transgenics might be competitive with wild-type untransformed plants in mercury-contaminated environment. These results were in concordance with the previous reports [6, 23], in which it was proposed that in the absence of substrate, MerA reduces the atmospheric O_2 to hydrogen peroxide (H_2O_2), which is toxic for cells. Hence, under mercury-free conditions, MerA expressed from transformed tobacco plants produced more H_2O_2, and in the presence of mercury, MerA bound tightly to Hg^{2+} and prevented cell-damaging side reactions.

Mercury Volatilization

A higher amount of Hg^0 evolved from the tissues of all the transgenic plants in comparison with wild-type control plants when the Hg volatilization study was performed with tissue culture plants. Apparently, the root system produced the highest mercury vapor, followed by leaves and stems, in both, the transgenic lines as well as in the wild-type control

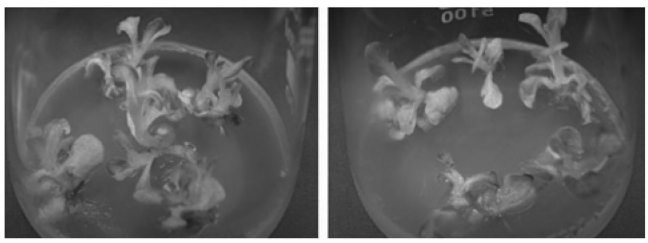

Fig. 6. Mercury response or tolerance assay.
Kanamycin-resistant *merA*-transformed tobacco shoots (showing 2 lines) derived from callus (after initial 3 months maintenance on MS medium) growing on MS medium amended with 100 μM and 120 μM $HgCl_2$. Wild-type tobacco shoots were not able to grow beyond 20 μM of $HgCl_2$ (data not shown).

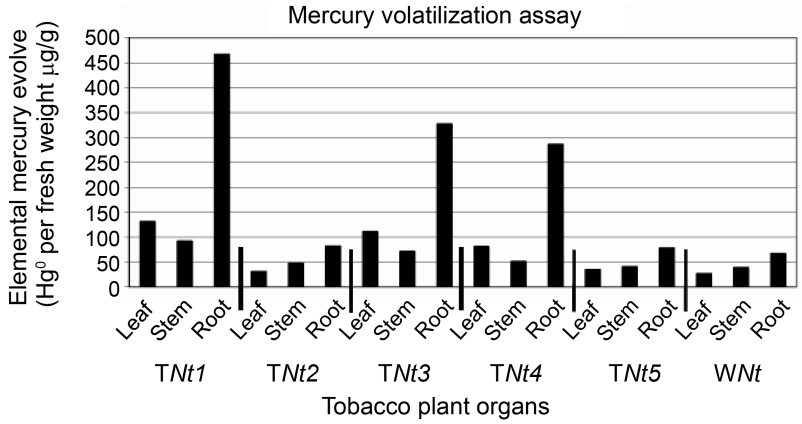

Fig. 7. Mercury volatilization assay.
Assays for Hg volatilization were performed in triplicates and the figure shows the amount of elemental Hg^0 released from transgenic tobacco plants grown in soilrite pots. Plant organs (leaves, roots, and stems) were harvested from kanamycin-resistant transgenic lines and wild-type (W*Nt*) plants, and immersed into $HgCl_2$ solution for the evolution of elemental Hg^0. The amount of elemental Hg^0 evolved was indicated as microgram of Hg^0 per gram of fresh plant tissue.

plants (data not shown). Mercury volatilization was assayed also at a later stage, using soilrite-grown plants. Since the soilrite-grown plants were too big to test, the leaves, stems, and roots were taken separately. Transgenic plants supplied with inorganic Hg attained a maximum rate of Hg volatilization in the first two days, with the rate declining to background levels after the third day. Outcomes of the vapor assay also showed that it was the root system of the transgenic plants that volatilize maximum mercury (Fig. 7). The enhanced mercury evolution from T*Nt1* plants was quite apparent in soilrite plants as compared with tissue culture plantlets. Maximum mercury vapor was released by the root system (Hg^0 per fresh weight, 467 µg/g), followed by the leaf (Hg^0 per fresh weight, 131 µg/g) and the stem (Hg^0 per fresh weight, 92 µg/g). The Hg^0 evolution figures were approximately 8, 3, and 5 times higher than the corresponding figures for the wild-type control plant, respectively. Contrary to that, T*Nt2* and T*Nt5* failed to show enhanced Hg^0 evolution; the amount of Hg^0 evolved from various organs of T*Nt2* and T*Nt5* were quite similar to that of the wild-type control. Apparently, the Hg^0 evolution capability of individual transgenic lines may differ greatly. Lines T*Nt1*, T*Nt3*, and T*Nt4* were highly resistant to inorganic mercury and showed good steady-state mRNA levels, but only T*Nt1* showed significant mercury volatilization whereas T*Nt3* and T*Nt4* were able to reduce a lesser amount of mercury.

Discussion

Wetland trees/plants expressing *merA* genes efficiently converted toxic Hg^{2+} to volatile Hg^0, and could be a solution to the dismal problem of mercury pollution by applying with enhanced phytoremediation [25]. Transgenic plants carrying the individual mercury metabolic genes can be crossed to create a universal mercury-removing plant for areas where methylmercury and ionic mercury pollution are simultaneously present [3, 11, 12, 19, 25]; the technology is still under development. Since tobacco is a fast-growing crop with large fresh mass, transgenic lines resistant to mercury could be used for the reclamation of a mercury-polluted region. We have characterized the *merA* gene encoding mercuric reductase from environmental *E. coli* strains that converts the toxic ionic mercuric derivative to the least toxic elemental form. The gene encoding mercuric reductase (*merA*) is a part of the *mer* operon and present on plasmid. Transformation of $HgCl_2$-sensitive DH5α strains of *E. coli* gave the same resistance pattern as the wild-type isolates; the maximum number of transformants were observed in the YR sample and the lowest for the KR sample. Expression of the bacterial *merA* gene in transgenic tobacco significantly increased Hg^{2+} tolerance in comparison with wild plants, and volatilized higher mercury from mercury-containing medium, as shown by other groups for transgenic *Arabidopsis* [6, 23, 24] and tobacco [12, 14, 21]. A bacterial *merA* gene enabled transgenic tobacco plants to grow well on 80–140 µM $HgCl_2$, concentrations that retarded the control wild tobacco plants [3]. When the resulting *merA* transgenic plants were grown in a medium placed with toxic levels of mercury, the presence of Hg^{2+} did not cause considerable morphological changes in the *merA* transgenics, and they grew normally, similar to the wild-type tobacco plants. Expression of *merA* was varied for the different transformed tobacco lines. We are aware that multigene integration in the plant genome could lead to the gene silencing phenomenon, and this might explain the lower expression level in some of the transformed lines.

Moreover, the non-ionic Hg evolution was directly proportional to the mRNA of the *merA* gene, and RT–PCR results provided evidence that the mercury detoxification observed in transgenic plants was due to the action of the introduced gene. Results of Western blot clearly indicated the expression of *merA* in the transgenic tobacco lines, and the mercury tolerance assay performed with inorganic mercury for the transgenics clearly indicated a 5–7 times higher tolerance towards inorganic mercury in comparison with wild-type control plants. These results suggest that *merA*-encoded mercurial reductase has abilities to reduce mercury toxicity. The bacterial native *merA* gene has been previously expressed *via* plastid genome [21], although this is the first time we are reporting the expression of a native *merA* gene *via* the nuclear genome of *Nicotiana tabacum*. The key findings of the study were the expression of bacterial native *merA* gene in transgenic tobacco plants without making any change in its gene sequence, and volatilization of increased amount of mercury (6–7 times higher than wild-type plant) from the transgenic tobacco plants. Based on the findings, we can say that the expression of native *merA* gene *via* the nuclear genome of transgenic tobacco has the potential of becoming a useful phytoremediation system in the near future for mercury-polluted areas. The placement of these improved plants around pollution sources and at their point of discharge and harvest could prevent toxic Hg^{2+} accumulation and transport from these locations. Air movement will dilute the transformed mercury (Hg^0) into nontoxic levels and remove it from these locations. This mercury will be re-oxidized in the atmosphere and return diluted to the terrestrial and marine sediments, bound to sulfur and carbon compounds. Ultimately, this should lead to a more natural distribution of mercury in the environment and lower the mercury concentration to a nontoxic level in polluted areas where it threatens wildlife and human populations.

Tobacco is a non-food and non-feed crop and is self-pollinated, minimizing transgene escape and contamination of the food chain. Furthermore, a cytoplasmic male sterility system for tobacco plants has been developed that inhibits formation of pollen. Thus, Hg phytoremediation using tobacco transgenic lines is an environmentally friendly approach. This study demonstrated that phytoremediation can be stretched beyond the natural physiology of the tobacco plant by introducing a bacterial native *merA* gene (without codon modification) with enhanced mercury detoxification capacity into tobacco plants. Our next aim is to ascertain the relationship between the level of expression of *merA* and the efficiency of mercuric ion reduction, and to conduct field trials of tobacco transgenics. Many transgenic plants have been made with modified/unmodified *merA*/*merB*/*merAB*, and a few transgenic trial studies have already been performed, and some are still under progress in greenhouses or on spiked soil under laboratory conditions, but none have been finally tested into fields and the efficiency of these transgenics is still doubtful.

A recent review by Ruiz and Daniell [22] presented a nice overview and comparison of the various strategies to generate plants for Hg phytoremediation through *merA/merB/merAB* engineering *via* the chloroplast or nuclear genome, and proves that expression of *mer* genes *via* chloroplast have more potential with limitation of transformation efficiency, but our findings are encouraging and inspirational. Although the mercury volatilization/tolerance assays we had performed here are confirmatory in nature, but, there is a long way to go for the industrial scale production of *merA* transgenics for mercury phytoremediation. Hence, for the successful completion of this compelling study and for the future directions, there may be further possibilities to improve the mercury volatilization bioassays of the transgenics by exposing to mercury for long-duration time periods (like several weeks or months), or growing the plants under spiked soil/field conditions. Mass-balance studies using atomic absorption spectrometry to measure the mercury content in different plant tissues including root and shoot could be helpful to address a better understanding of the absorption, accumulation, and translocation of mercury to plant leaves. These assays could provide an enhanced understanding of the phytoremediation capabilities of the transgenic plants. As we are aware that phytoremediation of a Hg-contaminated system is a complex and multifactorial issue, further research is needed to explore the real advantages of this method in Hg-polluted soil in field conditions.

There is further chance of improvement in mercury tolerance by modifying the *merA* gene, such as alteration in flanking sequences, codon usages optimization, and decreased percentage of G+C content. Currently, our research group is aggressively engaged in the final stage of development of a luminescence (*mer-lux*)-based biosensor for the detection of mercury ions in polluted water samples, and characterization of different arsenic resistance (*ars*) genes for the application of transgenic phytoremediation to arsenic pollution. In a recent report, Nagata *et al.* [20] has shown the integration of *merT* and *ppk* (mercury transporter and mercury chelator polyphosphate) into tobacco for the enhanced uptake and bioaccumulation of mercury; thus, in our next research endeavor, we are also contemplating to integrate more genes of bacterial origin (like *merT* and *ppk*) in the *merA* transgenic tobacco genome for enhanced phytoremediation.

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