# Bioremediation and Biodegradation

## Analysis of Transgenic Indian Mustard Plants for Phytoremediation of Metal-Contaminated Mine Tailings

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### ABSTRACT

**Transgenic Indian mustard [*Brassica juncea* (L.) Czern.] plants overproducing the enzymes γ-glutamylcysteine synthetase (ECS) or glutathione synthetase (GS) were shown previously to have increased levels of the metal-binding thiol peptides phytochelatins and glutathione, and enhanced Cd tolerance and accumulation. Furthermore, transgenic Indian mustard plants overexpressing adenosine triphosphate sulfurylase (APS) were shown to have higher levels of glutathione and total thiols. These results were obtained with a solution culture. To better examine the phytoremediation potential of these transgenics, a greenhouse experiment was performed in which the transgenics were grown on metal-contaminated soil collected from a USEPA Superfund site near Leadville, Colorado. A grass mixture used for revegetation of the site was included for comparison. The ECS and GS transgenics accumulated significantly ($P < 0.05$) more metal in their shoot than wild-type (WT) Indian mustard, while the APS plants did not. Of the six metals tested, the ECS and GS transgenics accumulated 1.5-fold more Cd, and 1.5- to 2-fold more Zn, compared with wild-type Indian mustard. Furthermore, the ECS transgenics accumulated 2.4- to 3-fold more Cr, Cu, and Pb, relative to WT. The grass mixture accumulated significantly less metal than Indian mustard: approximately 2-fold less Cd, Cu, Mn, and Zn, and 5.7-fold less Pb than WT Indian mustard. All transgenics removed significantly more metal from the soil compared with WT Indian mustard or an unplanted control. While WT did not remove more metal than the unplanted control for any of the metals tested, all three types of transgenics significantly reduced the soil metal concentration, and removed between 6% (Zn) and 25% (Cd) of the soil metal. This study is the first to demonstrate enhanced phytoextraction potential of transgenic plants using polluted environmental soil. The results confirm the importance of metal-binding peptides for plant metal accumulation and show that results from hydroponic systems have value as an indicator for phytoremediation potential.**

Metals are released into the environment at increasing rates by mining, industry, and agriculture, causing serious problems for environmental and human health (Lantzy and Mackenzie, 1979; Nriagu, 1979; Ross, 1994). In the USA alone, more than 50 000 metal-contaminated sites await remediation, many of them Superfund sites (Ensley, 2000). Common remediation methods include soil washing, excavation and reburial for metal-contaminated soils, and pump and treat systems for water (Glass, 1999). Presently, the U.S. remediation costs are $7 billion to $8 billion per year, approximately 35% of which involves remediation of metals (Glass, 1999, 2000).

A relatively new technology for remediation of metals involves the use of plants: phytoremediation. This tends to be a relatively inexpensive technology, since it is performed in situ and is solar-driven (Salt et al., 1995, 1998). Phytoremediation can be used in conjunction with other cleanup methods (e.g., to polish a partly cleaned site). Phytoremediation is gaining acceptance: for instance, the U.S. phytoremediation market for metals is expected to grow from $20 million per year at present to $150 million per year in 2005 (Glass, 1999, 2000).

Principal phytoremediation strategies for metals are stabilization and accumulation (Salt et al., 1998). Phytostabilization of metals may employ plants to reduce leaching, runoff, and erosion via stabilization of soil by plant roots, or metals may be transformed to less toxic forms (Berti and Cunningham, 2000). Accumulation of metals in shoot tissue, followed by harvesting of shoot biomass, is called phytoextraction (Blaylock and Huang, 2000). The harvested plant material may be used for non-food purposes; alternatively, it can be ashed followed by recycling of the metals or disposal in a landfill (Chaney et al., 2000). These various metal phytoremediation technologies are already being used effectively (Salt et al., 1998; Blaylock, 2000).

To further enhance the efficiency of metal phytoremediation, the following strategies may be used: (i) new suitable plant species may be identified in screening studies, (ii) agronomic practices may be optimized for maximal biomass production and metal uptake (Chaney et al., 2000), and (iii) selected species may be further bred for the desired property, via classical breeding or genetic engineering. Genetic engineering has the advantage that it is relatively fast and it is possible to introduce genes from other species.

Genetic engineering has already been used successfully to enhance plant metal tolerance and accumulation. This was achieved either by overproducing metal-chelating molecules such as citrate (de la Fuente et al., 1997), phytochelatins (Zhu et al., 1999a,b), metallothioneins (Evans et al., 1992; Hasegawa et al., 1997), or ferritin (Goto et al., 1999), or by overexpression of metal transporter proteins (Samuelsen et al., 1998; Arazi et al., 1999; Van der Zaal et al., 1999; Curie et al., 2000; Hirschi et al., 2000). Also, mercury volatilization and tolerance was achieved by introduction of a bacterial pathway (Rugh et al., 1996; Bizily et al., 1999, 2000). For recent

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Published in J. Environ. Qual. 32:432–440 (2003).

**Abbreviations:** APS, adenosine triphosphate sulfurylase; γ-ECS, γ-glutamylcysteine synthetase; GS, glutathione synthetase; WT, wild type.

reviews on phytoremediation of metals using transgenic plants, see Krämer and Chardonnens (2001) and Pilon-Smits and Pilon (2002).

The increase in metal accumulation as the result of these genetic engineering approaches is typically two- to threefold more metal per plant, which potentially enhances phytoremediation efficiency by the same factor. It is as yet not clear how applicable these transgenics are for environmental cleanup, since no field studies have been reported. Lab studies that have shown promising results were usually performed in hydroponic systems, using nutrient solution spiked with one metal of interest. This is quite different from a field situation where plants are often grown on marginal, aged soil, polluted with mixtures of metals.

The goal of this study was to better assess the phytoremediation potential of transgenic Indian mustard plants engineered to overproduce metal-binding peptides. Transgenics overexpressing the glutathione-synthesizing enzymes γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS) were previously shown to have enhanced Cd tolerance and accumulation (Zhu et al., 1999a,b). The levels of glutathione, phytochelatins, and total thiols in the ECS transgenics were 1.5- to 2.5-fold higher than in wild-type plants, both in the presence and absence of Cd (Zhu et al. 1999b), while the GS transgenics showed enhanced levels of glutathione (5-fold), phytochelatins (2-fold), and total thiols (2-fold), but only in the presence of Cd. In a different study, overexpression of the key enzyme for cysteine synthesis, APS, was shown to lead to increased levels of glutathione (2-fold) and total thiols (1.5-fold), and to enhanced Se tolerance and accumulation (Pilon-Smits et al., 1999). These results were all obtained with hydroponic systems. In the experiment described here, the same transgenics were tested for their phytoremediation potential in a greenhouse experiment using aged polluted soil containing a mixture of metals.

The soil used for this study was collected from a Superfund site south of Leadville, CO, along the Arkansas River. The metal-contaminated mine tailings on this site and many others in the area are the result of 150 years of mining. This particular soil was chosen to test the phytoremediation potential of transgenic Indian mustard plants because it contained suitable levels of several metals of interest, not to ultimately use these transgenics on the actual site. However, plant-based reclamation of the area is underway: Dr. Sally Brown (University of Washington) and coworkers in collaboration with the USEPA are currently doing a revegetation project on the contaminated site (S. Brown, personal communication, 2000). The seed mixture used to phytostabilize the site in the revegetation project was also used for the greenhouse experiment described here, for a comparison with the transgenic and wild-type Indian mustard plants.

## MATERIALS AND METHODS

### Plant Material

Indian mustard (Accession no. 173874) seeds were obtained from the North Central Regional Plant Introduction Station, Ames, IA, and propagated. The untransformed (wild-type) seeds were used for this study, as well as three types of transgenics, obtained by transformation of this genotype with different DNA constructs. Transgenic APS plants were obtained by overexpression of the mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.] *APS1* cDNA including its own chloroplast transit sequence, under the control of the CaMV 35S promoter, as described by Pilon-Smits et al. (1999). Transgenic ECS plants were obtained by expression of the *Escherichia coli gshI* gene, fused to a pea chloroplast transit sequence and driven by the *Cauliflower mosaic virus* 35S promoter with a double enhancer sequence, as described by Zhu et al. (1999b). Transgenic GS plants were obtained as described by Zhu et al. (1999a), by expression of the *E. coli gshII* gene, driven by the double-enhanced 35S CaMV promoter. For this study, one representative line was chosen for each type of transgenic (i.e., APS8, cytGS7, and cpECS4). For all lines seed batches were used that are homozygous for the introduced gene.

A seed mixture used by the USEPA for revegetation of the Leadville site was included in the study, for comparison. This mixture, predominantly comprised of grasses, included the following species: tall wheatgrass [*Elytrigia elongata* (Host) Nevski], Canadian wild rye (*Elymus canadensis* L.), Canadian bluegrass (*Poa compressa* L.), redtop (*Agrostis stolonifera* L.), tufted hairgrass [*Deschampsia cespitosa* (L.) P. Beauv.], western wheatgrass [*Pascopyrum smithii* (Rydb.) Á. Löve], smooth brome (*Bromus inermis* Leyss.), and alsike clover (*Trifolium hybridum* L.).

### Soil

Metal-contaminated soil was obtained from a mine tailing along the upper Arkansas River (USEPA Site QN, 1220 $m^3$ of tailing), about 10 miles south of Leadville. The average metal concentrations reported for the entire site are 640 mg $kg^{-1}$ Cu, 2400 mg $kg^{-1}$ Pb, 2700 mg $kg^{-1}$ Mn, 7800 mg $kg^{-1}$ Zn, and 3.56 mg $kg^{-1}$ leachable (toxicity characteristic leaching procedured [TCLP]) Cd (USEPA, 1997).

### Soil Collection and Experimental Design

Topsoil (0- to 30-cm depth) was collected from the southern edge of the QN site, from an unvegetated area bordering on a vegetated section. To test the requirements to make this soil amenable to plant growth, a greenhouse pilot experiment was done, testing plant growth after addition of different amounts of lime and compost. Without amendments no plant growth could be achieved. Vigorous plant growth could be obtained with 1:10 compost to soil (v/v), and addition of lime to bring the soil up to a pH of 6 (from an original pH 3). The compost used (EKO composted forest product) was obtained from Richlawn Turf Food (Platteville, CO). The lime used was Western Lawn and Garden Lime (Western Lime Corp., West Bend, WI), containing 61% calcium hydroxide and 39% magnesium hydroxide, with a $CaCO_3$ equivalence of 136 and a neutralization value of $100^+$. Incidentally, a similar amendment was used in the USEPA revegetation project on the site. After amendment, the soil was homogenized and distributed over 18-cm-diameter (2 L) plastic pots, and seeds were sown directly onto the soil. Before sowing, soil core samples were taken from each pot and pooled per three pots. Thus, three samples per 10 pots were obtained ($T_0$ samples), which were analyzed for metal concentration as described below.

The following six treatments were compared: an unplanted control, wild-type (WT) Indian mustard, transgenic APS Indian mustard, transgenic GS Indian mustard, transgenic ECS Indian mustard, and a mix of grasses used by the USEPA for

phytostabilization on the site (see above). The Indian mustard pots were thinned to 10 seedlings per pot after one week; the grass mixture pots were kept at maximal plant density (i.e., one or two plants per $cm^2$, comparable with lawn density). Ten replicate pots were used per treatment. The plants were grown in the greenhouse for 14 weeks at 22°C, with daily watering and no fertilization, and under natural light (December 2000–March 2001, Fort Collins, CO). The plants were harvested after 14 weeks, on the first signs of senescence in the Indian mustard plants. On the day of harvest the collective shoots were harvested from each pot and dried for 2 d at 80°C. The total shoot biomass (dry weight) was then determined for each pot, and the dried shoot material was further analyzed for metal content as described below. In addition, root and soil samples were taken from each pot as follows. For each pot the total soil material was collected and air-dried. Then the soil was broken up and roots were harvested by hand. The root material was washed in water and dried for 2 d at 80°C for elemental analysis. For each pot, the soil was then homogenized by hand, and one approximately 100-g sample was collected for elemental analysis as described below.

### Metal Analyses

In preparation for acid digestion, dried plant material (the entire shoot or root tissue collected from each pot) was ground to a powder with a Wiley mill. The soil samples were sieved through a fine mesh (40 mesh, no. 11156; Ace Hardware, Oak Brook, IL) to remove residual plant material. Acid digestion was performed on the plant and soil samples according to the method of Zarcinas et al. (1987) as described by Zhu et al. (1999a). Total metal concentrations in the digests were determined with inductively coupled plasma atomic emission spectrometry (ICP–AES; Thermo Jarrell Ash, Franklin, MA) according to the method of Fassel (1978).

Statistical analyses were performed with the SAS statistical software program JMP-IN (SAS Institute, 1996). For all measurements the mean and standard error of the mean ($n = 10$) are shown in the figures. To statistically compare each pair of means, $t$ tests were performed. Statistically significant ($\alpha = 0.05$) groups are shown with different letters in the figures.

## RESULTS

The objective of this study was to assess the phytoremediation potential of transgenic Indian mustard plants overexpressing γ-glutamylcysteine synthetase (ECS plants), glutathione synthetase (GS plants), or adenosine triphosphate sulfurylase (APS plants), each shown previously to overproduce metal-binding thiol peptides. The transgenics were sown on metal-polluted soil collected from a Leadville Superfund site, together with wild-type (WT) Indian mustard for comparison, as well as a grass mixture and an unplanted control. After 14 weeks of growth on the metal-polluted soil the plants were harvested and shoot metal concentrations were compared pairwise ($t$ test, $\alpha = 0.05$) between the different treatments (Fig. 1). Compared with WT Indian mustard the ECS and GS transgenics contained higher shoot concentrations of Cd (+50%) and Zn (+45% for GS and +93% for ECS). Furthermore, the ECS transgenics had higher levels of Cr (+170%), Cu (+140%), and Pb (+200%), relative to WT. There were no significant differences in shoot metal concentration between the APS transgenics and WT Indian mustard. The grass

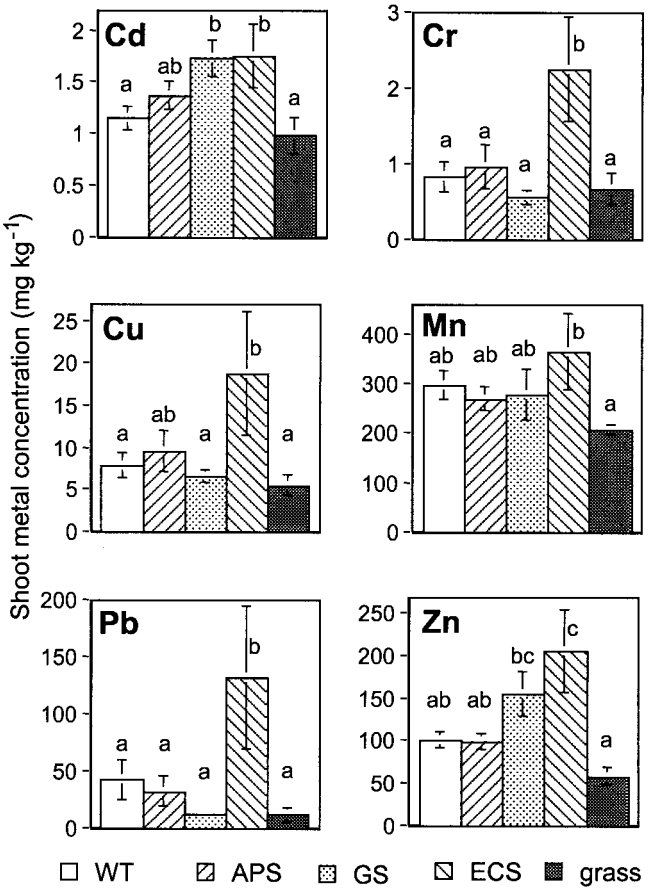


**Fig. 1. Shoot metal concentrations for the five planted treatments at harvest. Shown values are the means and standard errors of 10 replicate pots (shoot material was pooled for each pot, dried, homogenized, and analyzed for metals). WT, wild-type Indian mustard; APS, adenosine triphosphate sulfurylase–overexpressing Indian mustard; GS, glutathione synthetase–overexpressing Indian mustard; γ-ECS, γ-glutamylcysteine synthetase–overexpressing Indian mustard. The letters above the bars indicate statistically significant groups ($t$ test, compairing each pair, $\alpha = 0.05$).**

mixture contained the lowest shoot metal levels for all metals, but the concentrations in the grass were not significantly lower than those of WT Indian mustard.

There were no apparent differences in metal tolerance between the transgenics and WT Indian mustard. No chlorosis or necrosis were observed in any of the plant types. The shoot biomass per pot (or per plant) showed no significant differences between the various transgenic Indian mustard lines and WT (Fig. 2A). Grass shoot biomass per pot was 30% lower than Indian mustard shoot biomass; however, this difference was only significant ($P < 0.05$) between grass and GS Indian mustard. By multiplying the shoot metal concentration (Fig. 1) with the total shoot biomass per pot (Fig. 2A) the total amount of metal in the plant shoot per pot was calculated as a measure of phytoextraction efficiency. Since the shoot biomass of all Indian mustard plant types was the same, their shoot metal accumulation per pot (not shown) was a direct reflection of shoot metal concentrations. However, since the grass mixture contained somewhat lower shoot metal concentrations as well as somewhat smaller shoot biomass, the overall

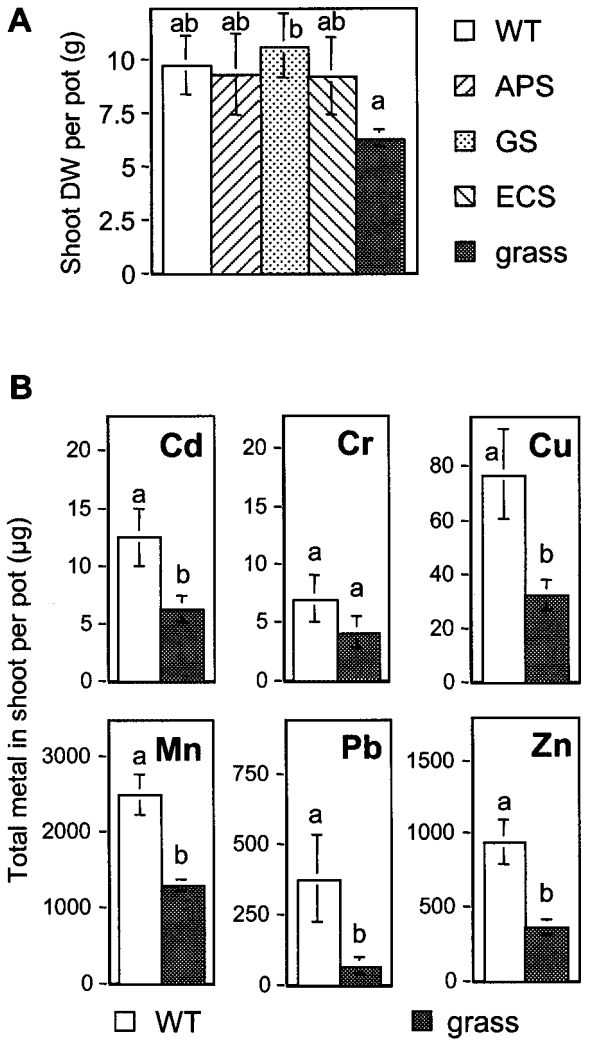


**Fig. 2. (*A*) Plant shoot dry weight per pot for the five planted treatments. (*B*) Total metal in shoot per pot. Shown values are the means and standard errors of 10 replicate pots (shoot material was pooled from each pot). WT, wild-type Indian mustard; APS, adenosine triphosphate sulfurylase–overexpressing Indian mustard; GS, glutathione synthetase–overexpressing Indian mustard; γ-ECS, γ-glutamylcysteine synthetase–overexpressing Indian mustard. The letters above the bars indicate statistically significant groups (*t* test, compairing each pair, α = 0.05).**

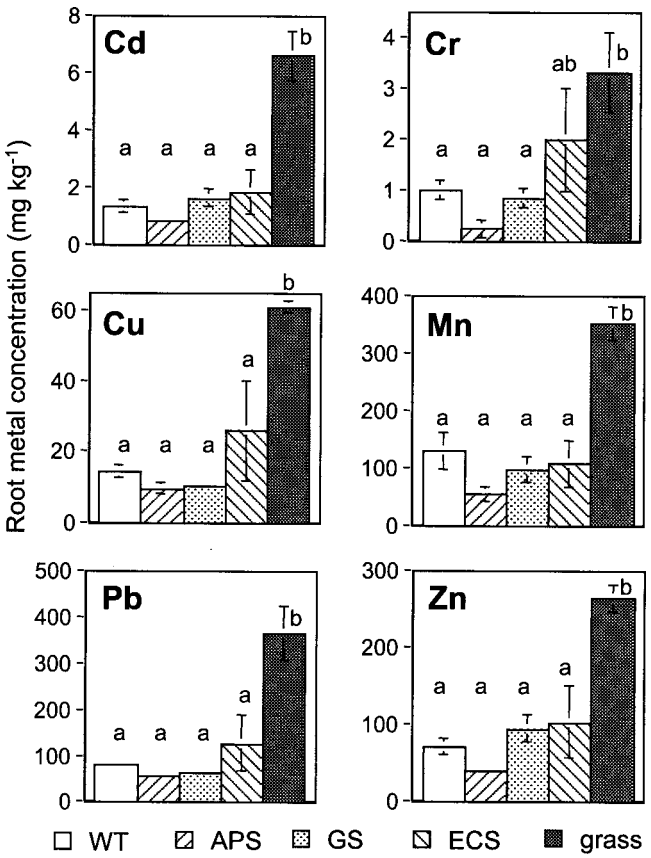


**Fig. 3. Root metal concentrations for the five planted treatments at harvest. Shown values are the means and standard error of 10 replicate pots (one root sample was taken per pot). WT, wild-type Indian mustard; APS, adenosine triphosphate sulfurylase–overexpressing Indian mustard; GS, glutathione synthetase–overexpressing Indian mustard; γ-ECS, γ-glutamylcysteine synthetase–overexpressing Indian mustard. The letters above the bars indicate statistically significant groups (*t* test, compairing each pair, α = 0.05).**

shoot metal accumulation per pot was significantly smaller for the grass mixture than for WT Indian mustard for all metals tested except Cr (Fig. 2B). Wild-type Indian mustard accumulated approximately 2-fold more Cd, Cu, Mn, and Zn than the grass, and 5.7-fold more Pb.

The root metal concentrations were not significantly different between any of the transgenics and WT, although Cr and Cu levels were 1.6- to 2-fold higher in ECS plants (Fig. 3). Levels of all six metals were higher in the grass roots than in the Indian mustard roots ($P < 0.05$). From visual observation, root biomass was much higher for the grass than for Indian mustard. Unfortunately, this could not be quantified since it proved too difficult to quantitatively recover all Indian mustard roots from the soil; the roots were very fine (<1 mm in diameter) and could not be effectively separated from the bigger soil particles on a large scale.

The root to shoot metal concentration ratios were similar for all transgenic Indian mustard lines and WT (Table 1). Grass, on the other hand, showed higher root to shoot ratios than Indian mustard for all six metals ($P < 0.05$), indicating less metal translocation to the shoot. Indian mustard apparently translocates metals and accumulates them in the shoot, while these grasses tend to accumulate them in the root. The degree to which the six metals were translocated followed the same pattern in both species, that is, Pb < Cu < Cr < Cd < Zn < Mn (Table 1).

Soil samples were taken from all treatments at the beginning and end of the experiment, acid-digested, and analyzed for total metal concentrations (Fig. 4). Soil collected from pots in which any of the three types of transgenics had grown in general showed lower metal concentrations than soil in which the WT Indian mustard plants had grown, or soil from the unplanted control. Soils treated with APS and GS Indian mustard or the grass mixture contained lower levels of all six metals tested, compared with WT Indian mustard–treated soil ($P < 0.05$). The ECS Indian mustard–treated soil had significantly lower levels of Pb and Zn than WT-treated soil ($P < 0.05$); their levels of Cd, Cr, and Cu were

**Table 1. Ratio of root to shoot metal concentration in wild-type Indian mustard (WT), adenosine triphosphate sulfurylase–overexpressing Indian mustard (APS), glutathione synthetase–overexpressing Indian mustard (GS), γ-glutamylcysteine synthetase–overexpressing Indian mustard (ECS), or a grass mixture (grass). A high ratio corresponds with a low level of root-to-shoot metal translocation. Means and standard errors are shown ($n = 10$ pots, using pooled shoot biomass for each pot). Letters indicate statistically significant groups ($t$ test, compairing each pair, $\alpha = 0.05$).**

| | Ratio of root to shoot metal concentration | | | | |
|---|---|---|---|---|---|
| **Metal** | **WT** | **APS** | **GS** | **ECS** | **Grass** |
| **Cd** | 1.5 ± 0.3a | 0.7 ± 0.1a | 1.1 ± 0.2a | 0.9 ± 0.2a | 6.5 ± 0.6b |
| **Cr** | 2.3 ± 0.6a | 0.3 ± 0.2a | 1.4 ± 0.4a | 1.7 ± 0.7a | 8.3 ± 1.8b |
| **Cu** | 3.0 ± 0.6a | 1.6 ± 0.5a | 1.9 ± 0.4a | 2.1 ± 0.8a | 15.2 ± 2.6b |
| **Mn** | 0.5 ± 0.1a | 0.2 ± 0.04a | 0.4 ± 0.1a | 0.4 ± 0.1a | 1.7 ± 0.2b |
| **Pb** | 12.6 ± 4.6a | 10.6 ± 3.8a | 11.8 ± 2.8a | 6.5 ± 2.9a | 171.7 ± 43.0b |
| **Zn** | 0.8 ± 0.2a | 0.4 ± 0.1a | 0.7 ± 0.1a | 0.5 ± 0.1a | 4.9 ± 0.5b |

lower than WT-treated soil as well, but these differences were not significant ($0.10 < P < 0.17$).

The transgenics significantly reduced soil metal concentrations, in contrast to wild-type plants. While the metal concentrations in the WT Indian mustard–treated soils at harvest were not significantly different from the metal levels in the unplanted control soil, the concentrations of all six metals were significantly lower in APS Indian mustard–treated, GS Indian mustard–treated, and grass-treated soils ($P < 0.05$, Fig. 4). The ECS Indian mustard–treated soils showed intermediate metal levels between soils treated with the other transgenics and the unplanted control.

When soil metal concentrations were compared between the beginning and end of the experiment the APS and GS transgenics as well as the grass mixture significantly decreased soil concentrations of all six metals ($P < 0.05$); the ECS transgenics lowered soil Cd, Pb, and Zn levels (Fig. 4). In contrast, WT Indian mustard–treated soils at harvest did not differ in metal concentration from the initial levels, except for Cd. Surprisingly, the unplanted control soil showed a significant decrease in Cd and Zn compared with the initial soil, which may have been due to leaching or to adsorption to the pot (not analyzed).

The percentage of metal removed from the soil over the course of the experiment varied from 0 to 14% for most metals, but from 12 to 25% for Cd (Fig. 5). The percentage of metal removed by the APS and GS transgenics or the grass mixture was significantly higher com-

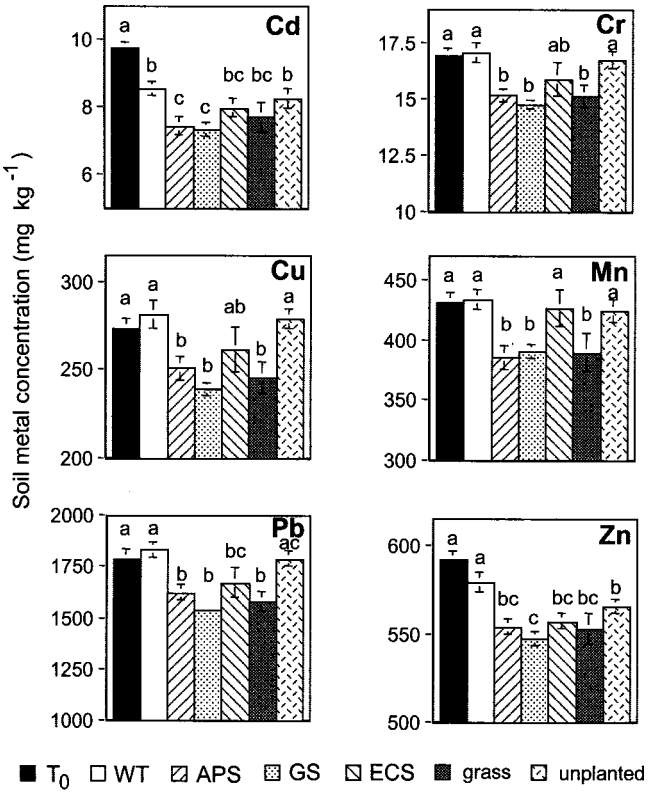


**Fig. 4. Soil total metal concentrations at the beginning of the experiment ($T_0$) and at harvest. Shown values are the means and standard error of 10 replicate pots (at harvest one sample was taken per pot after soil homogenization). Note: if error bars are not visible they were too small to be plotted. WT, wild-type Indian mustard; APS, adenosine triphosphate sulfurylase–overexpressing Indian mustard; GS, glutathione synthetase–overexpressing Indian mustard; γ-ECS, γ-glutamylcysteine synthetase–overexpressing Indian mustard. The letters above the bars indicate statistically significant groups ($t$ test, compairing each pair, $\alpha = 0.05$).**

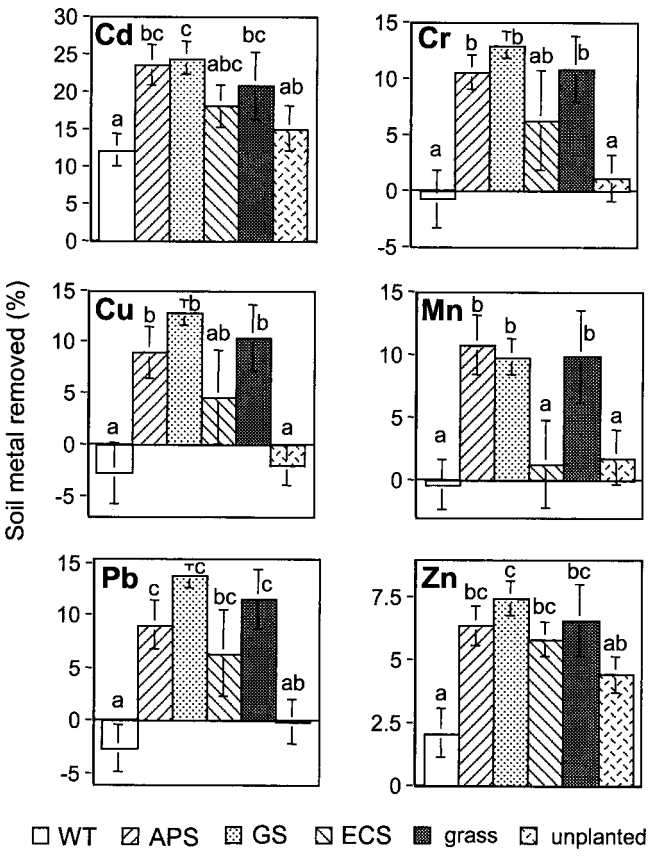


**Fig. 5. Fraction of soil total metal removed by each treatment, expressed as [(initial metal concentration − final metal concentration)/initial metal concentration] × 100%. Shown values are the means and standard errors of 10 replicate pots (after harvest one sample was taken from each pot after soil homogenization). WT, wild-type Indian mustard; APS, adenosine triphosphate sulfurylase–overexpressing Indian mustard; GS, glutathione synthetase–overexpressing Indian mustard; γ-ECS, γ-glutamylcysteine synthetase–overexpressing Indian mustard. The letters above the bars indicate statistically significant groups ($t$ test, compairing each pair, $\alpha = 0.05$).**

pared with WT Indian mustard for all six metals ($P < 0.05$). The ECS transgenics removed more Pb and Zn than WT; removal of Cd, Cr, and Cu by ECS was also somewhat higher, but this difference was not significant. While metal removal by WT Indian mustard was not different from metal loss in the unplanted control, the APS and GS Indian mustard transgenics and grass removed significantly more of all metals than the unplanted control ($P < 0.05$); the ECS transgenics removed intermediate levels of metals between the other transgenics and the unplanted control.

## DISCUSSION

Transgenic Indian mustard plants engineered to produce more of the metal-binding peptides glutathione and phytochelatins accumulated significantly more metal in their shoot than wild-type Indian mustard (Fig. 1 and 2), and removed more metal from contaminated soil compared with wild-type or an unplanted control (Fig. 4 and 5). The significance of this finding is that it is the first to demonstrate an enhanced capability of transgenic plants to phytoextract environmental soil containing a mixture of metals, compared with their wild-type relatives.

The finding that these ECS and GS plants accumulated more Cd in their shoots is in agreement with results from previous hydroponic studies (Zhu et al., 1999a,b). Thus, hydroponic systems appear to be a reasonable model to predict the metal phytoextraction potential of plants. In addition to Cd, the transgenics showed enhanced accumulation of several other metals. Based on total shoot accumulation (biomass × shoot metal concentration) the ECS plants showed the greatest increase in phytoextraction capacity relative to WT Indian mustard ($P < 0.05$ for Cd, Cr, Cu, Pb, and Zn), followed by GS plants ($P < 0.05$ for Cd and Zn). Although these results are based on only one transgenic line per construct, they suggest that of the overexpressed enzymes tested here, γ-ECS is the most rate-limiting for shoot metal accumulation. The GS enzyme appears to be limiting to a lesser extent. This is in agreement with earlier studies showing that γ-ECS is the most rate-limiting enzyme for glutathione production, while GS can become colimiting under metal stress, when γ-ECS is upregulated (Zhu et al., 1999b; Foyer et al., 1995; Noctor et al., 1998). It should be noted that when metal removal from soil is used as a criterium, the GS and APS transgenics appear to have been the most efficient metal phytoremediatiors rather than the ECS transgenics. Based on the available data it cannot be explained why the APS Indian mustard–treated soil lost more metals than the ECS Indian mustard–treated soil (Fig. 4 and 5) despite the lower shoot metal levels in the APS plants (Fig. 1). It is possible that the APS plants stored more metals in their roots than ECS plants; this cannot be ruled out since root biomass could not be analyzed.

Since the thiol-overproducing ECS and GS transgenics showed enhanced accumulation of Cd, these studies confirm a role for phytochelatins and/or glutathione in Cd sequestration. Phytochelatins may play a role in tolerance and accumulation of other metals as well. This has been shown most convincingly for As, which was reported to be bound and detoxified by phytochelatins in plants (Pickering et al., 2000; Schmöger et al., 2000). Although many other metals including Cu, Zn, and Pb induce phytochelatin synthesis and can often bind to phytochelatins in vitro, more research is needed to determine any phytochelatin involvement in vivo (Cobbett and Goldsbrough, 2000). The observation that the transgenics in this study showed enhanced accumulation of Zn and to a lesser extent Cr, Cu, and Pb sheds some new light on the possible involvement of phytochelatins and glutathione in sequestration of these metals.

In the hydroponic studies the ECS and GS transgenics showed better growth in the presence of Cd than wild-type plants; however, in the present study the transgenics and wild-type grew equally well. Most likely, the plants were not metal-stressed to the same extent in the present study. Indeed, the plants showed no signs of metal toxicity and the metal levels in the plant tissues were not excessive. For instance, the Mn levels in the shoots of these plants were around 300 mg $kg^{-1}$ dry wt., whereas the critical toxicity level for plants (equal to the plant metal concentration where the metal starts to be toxic, giving 10% reduction in dry matter production) tends to be between 200 and 1400 mg $kg^{-1}$ dry wt. (Marschner, 1995). The Cu and Zn levels were around 10 and 150 mg $kg^{-1}$ dry wt., respectively, while the critical toxicity level for Cu is 20 to 30 mg $kg^{-1}$ dry wt. and for Zn is 100 to >300 mg $kg^{-1}$ dry wt. (Marschner, 1995). The addition of lime and compost to the soil was apparently very effective in making the metals less bioavailable and therefore less toxic; this is a known effect (Marschner, 1995). The chemical form of lime used (calcium and magnesium hydroxides) is expected to be especially effective at reducing metal bioavailability. This may also explain the fairly low shoot metal concentrations observed in the plants.

The amount of metal extracted by these plants in one crop was fairly small (Fig. 2B). A possible reason for the fairly low plant metal accumulation relative to soil metal levels is that the metals were very efficiently bound by the compost and made less bioavailable by the lime. Also, the bioavailability of the metals in this soil may have been relatively low in the first place, since this is aged soil and some of the bioavailable fraction may have already leached out on the site before soil collection. Some leaching on the site is suggested by the soil metal distribution, since metal levels tended to increase with soil depth (Zn levels were 8-fold higher in the 18- to 30-cm layer than in the 0- to 12-cm layer; Cu was 4-fold higher, and Cd 1.5-fold, while Cr, Pb, and Mn showed the same concentration throughout; J. Christner, personal communication, 2001).

During the course of the 14-week experiment 5% (Zn) to 25% (Cd) of the metals were removed from the soil (Fig. 5), which is probably as much as can be expected. For comparison, three crops of the metal hyperaccumulator *Thlaspi caerulescens* J. Presl & C. Presl removed 43% of Cd and 7% of Zn from an industrially contaminated soil over 391 d (Lombi et al., 2001). In a

different study, Indian mustard supplied with EDTA removed 35 to 53% of Pb from two industrial soils (Blaylock, 2000).

During the greenhouse pot experiment, the unplanted soil lost significant amounts of Cd and Zn, but not of Cr, Cu, Mn, or Pb, indicating that Cd and Zn were more prone to either leaching or adsorption to the pot than the other metals. A relatively high mobility and bioavailability for Cd and Zn is suggested by their shoot concentration factor (shoot to soil concentration ratio): relative shoot concentration factors for several plant species were reported to be Pb < Cr < Cd < Zn (Ross, 1994). In the present study, a similar order was observed (Pb < Cu < Cr < Cd < Zn < Mn) when the root–shoot translocation factors of the six metals were compared; this order was the same for both Indian mustard and the grass species.

Judging from plant metal accumulation in root and shoot, the grasses appear to be more suitable for use in metal phytostabilization, while Indian mustard is better for metal phytoextraction. The grasses trapped the metals in or on their roots, which had higher metal concentrations than Indian mustard roots, and a higher biomass. As a result, the grasses removed as much metal from the soil particles as Indian mustard, but not in easy to harvest plant parts. Indian mustard was a more efficient phytoextractor, accumulating 2- to 5.7-fold more metal in its shoot biomass per pot, compared with the grass. Of course the efficiency of these different species will depend greatly on climate, therefore this comparison cannot be extrapolated to every field situation.

Although the shoot metal levels observed in the present study were moderate, the plant material would not be suitable for animal consumption. The Indian mustard shoots contained too high Cd and Pb levels, and the grass shoot Cd levels were too high to be used as feed for domestic animals (the maximal acceptable levels are 0.5 mg $kg^{-1}$ for Cd and 30 mg $kg^{-1}$ for Pb, respectively; J. Trlica, personal communication, 2001). Still, occasional grazing by wildlife would probably not pose a significant health hazard as long as the rest of the animals' diet is low in metals.

The results presented here confirm the importance of the metal binding thiols phytochelatins and glutathione for metal sequestration, and hence, phytoremediation. The ECS and GS transgenics showed enhanced phytoextraction potential compared with untransformed plants, as judged from their shoot metal accumulation during the greenhouse pot experiment. The question remains how these plants will perform in a real phytoremediation setting. To answer this question, the ultimate test would be a field phytoremediation experiment. This has not been done but may be feasible, if accompanied by an appropriate risk assessment study (Glass, 1997). Based on the results from this study, the GS and ECS transgenics are expected to be 1.5- to 3-fold more efficient for phytoextraction compared with their wild-type relative. Since WT Indian mustard is one of the most popular plants for metal phytoextraction (Blaylock et al., 1997) these transgenics with enhanced phytoextraction capacity may prove to perform well enough to be used in commercial phytoremediation. Another approach to further enhance the phytoextraction capacity of Indian mustard or other plants is the addition of synthetic chelators to the soil. For instance, addition of EDTA was shown to lead to an increase in shoot Pb concentration from 28 to 785 mg $kg^{-1}$ (Blaylock et al., 1997). In comparison, the genetically engineered ECS plants accumulated 135 mg $kg^{-1}$, relative to 45 mg $kg^{-1}$ for the WT Indian mustard. Thus, addition of synthetic chelators may be more effective than this biotechnological approach. On the downside of using chelators, addition of chelators may lead to enhanced metal leaching into the ground water due to the enhanced bioavailability (Lombi et al., 2001). Very high metal concentrations (1–2%) during phytoextraction can also be obtained without addition of chelators if metal hyperaccumulator plants are used (Robinson et al., 1998; McGrath et al., 2000). Unfortunately, these species are generally slow growers and produce little biomass. Only the hyperaccumulator *Alyssum bertolonii* Desv. subsp. *scutarinum* Nyár. is showing promise thus far, for phytomining of Ni (Chaney et al., 2000).

Any eventual application of these Indian mustard transgenics for phytoremediation will be restricted by the natural limitations of the species. However, the same biotechnological approach used for Indian mustard may also be used to create transgenics of other plant species, adapted to different environmental conditions. The production of metal-binding peptides and thus plant phytoextraction capacity may be further enhanced by combined ECS and GS overexpression in double-transgenic plants. This possibility will be explored in further studies.

## CONCLUSIONS

The results presented here suggest that overproduction of the metal-binding thiols glutatione and phytochelatins is a promising strategy for the production of plants with enhanced metal phytoremediation properties. The thiol-overproducing transgenic ECS and GS Indian mustard plants showed enhanced phytoextraction capacity compared with untransformed Indian mustard plants, as judged from total shoot metal accumulation. The ECS and GS transgenics contained higher shoot metal concentrations than wild-type, and produced the same shoot biomass. As a result, the total shoot metal accumulation of the ECS and GS transgenics were 1.5-fold higher for Cd, and 1.5- to 2-fold higher for Zn, compared with wild-type Indian mustard. Furthermore, the ECS transgenics accumulated 2.4- to 3-fold more Cr, Cu, and Pb, relative to WT. There were no significant differences in metal accumulation between the APS transgenics and WT Indian mustard. Due to both lower shoot metal levels as well as lower shoot biomass, the grass mixture accumulated less metal than the Indian mustard plants: approximately 2-fold less Cd, Cu, Mn, and Zn, and 5.7-fold less Pb than WT Indian mustard.

The transgenic Indian mustard plants reduced the soil metal concentrations more than WT Indian mustard:

while WT did not remove more metal than the unplanted control for any of the metals tested, all three types of transgenics significantly reduced the soil metal concentration, and removed between 6% (Zn) and 25% (Cd) of the soil metal. These results confirm earlier experiments performed in hydroponic systems with Cd, showing that results from hydroponic systems have value as an indicator for phytoextraction potential. It also confirms the importance of metal-binding peptide thiols for plant phytoextraction capacity, not only for Cd but also for other metals, and not only for single metals but also for mixtures of metals, even in aged soils from polluted sites. These results are of significance for environmental remediation, since thousands of metal-contaminated sites await cleanup and phytoremediation offers a cost-effective alternative for this purpose.

## ACKNOWLEDGMENTS

The authors would like to thank Jan Christner (URS Operating Services, Inc.) and Mike Zimmerman (USEPA, Denver, CO) for site information, donation of revegetation seed mixture, and advice. We also thank Rufus Chaney (USDA-ARS, Beltsville, MD), Sally Brown (Univ. of Washington, Seattle) and Joe Trlica (Colorado State Univ., Fort Collins) for helpful information and advice, and Gulnara Garifullina, Jeff Holynski, and Stormy Lindblom for technical assistance. This project was funded by an Environmental Protection Agency Research Grant (no. G8A11586), awarded to Norman Terry and Elizabeth A.H. Pilon-Smits, and a National Science Foundation Career Development Grant (no. 9982432) awarded to Elizabeth A.H. Pilon-Smits.

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