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HEAVY METAL TOLERANCE AND ACCUMULATION IN INDIAN MUSTARD (*BRASSICA JUNCEA* L.) EXPRESSING BACTERIAL γ-GLUTAMYLCYSTEINE SYNTHETASE OR GLUTATHIONE SYNTHETASE

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The overexpression of either γ-glutamylcysteine synthetase (γ-ECS) or glutathione synthetase (GS) in* Brassica juncea *transgenics was shown previously to result in higher accumulation of glutathione (GSH) and phytochelatins (PCs), as well as enhanced Cd tolerance and accumulation. The present study was aimed at analyzing the effects of γ-ECS or GS overexpression on tolerance to and accumulation of other metal/loids supplied individually in agar medium (seedlings) or in hydroponics (mature plants). Also, as pollution in nature generally consists of mixtures of metals, glutamylcysteine synthetase (ECS) and GS seedlings were tested on combinations of metals. Compared to wild-type plants, ECS and GS transgenics exhibited a significantly higher capacity to tolerate and accumulate a variety of metal/loids (particularly As, Cd, and Cr) as well as mixed-metal combinations (As, Cd, Zn/As, Pb, and Zn). This enhanced metal tolerance and accumulation of the ECS and GS transgenics may be attributable to enhanced production of PCs, sustained by a greater availability of GSH as substrate, as suggested by their higher concentrations of GSH, PC2, PC3, and PC4 as compared to wild-type plants. Overexpression of GS and γ-ECS may represent a promising strategy for the development of plants with an enhanced phytoremediation capacity for mixtures of metals.

KEY WORDS: transgenic, metal, metalloid, tolerance, accumulation, thiols

INTRODUCTION

Heavy metals and metalloids such as cadmium (Cd), chromium (Cr), molybdenum (Mo), zinc (Zn), manganese (Mn), lead (Pb), arsenic (As), and nickel (Ni) are released into the environment by many anthropogenic activities including industrial processes and agricultural practices (Pilon-Smits, 2005). Although regulatory steps have been implemented to reduce the discharge of contaminants into soils and waters, they are not enough to control heavy metal pollution worldwide (Zayed and Terry, 2003).

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Metal-contaminated substrates can be recovered using *exsitu* conventional remediation methods such as soil washing, excavation, and reburial (Ghosh and Singh, 2005; Pilon-Smits, 2005). Because of the prohibitive cost of these methods, in the last decades attention has been diverted toward the development of cost-effective alternative/complementary biological cleanup techniques such phytoremediation, which employs plants and their associated rhizosphere microrganisms to extract, degrade, or stabilize pollutants (Salt, Smith, and Raskin, 1998; Garbisu and Alkorta, 2001; Pilon-Smits, 2005).

Plants suitable for phytoremediation may be genetically engineered to enhance their ability to tolerate and accumulate heavy metals *via* the overexpression of genes encoding rate-limiting enzymes involved in metal tolerance and accumulation (Clemens, 2001; Clemens, Palmgren, and Krämer, 2002). Many successful examples of such a molecular approach are reported in the literature (for a review, see Pilon-Smits and Pilon, 2002). One approach involves the overproduction of metal chelators such as the thiol-rich peptides described below.

Plants have evolved a number of mechanisms to cope with heavy metal stress. These include the synthesis of the S-rich metal chelators glutathione (GSH) and phytochelatins (PCs) (Hall, 2002; Gasic and Korban, 2006). GSH, the most abundant low-molecular-weight thiol compound in plants (Bergmann and Rennenberg, 1993; Hell, 1997), is synthesized through a two-step ATP-dependent enzymatic pathway (Noctor *et al*., 2002). In the first reaction, γ-glutamylcysteine (γ-EC) is formed from glutamate and cysteine by γ-glutamylcysteine synthetase (γ-ECS) (Hell and Bergmann, 1990), which is encoded by the *gshI* gene (May and Leaver, 1994). Subsequently, GSH is synthesized by the ligation of γ-EC and glycine in the reaction catalyzed by glutathione synthetase (GS), which is encoded by the *gshII* gene (Wang and Oliver, 1996).

Glutathione plays a central role in protecting plants from environmental stresses, including oxidative stress, xenobiotics, and some heavy metals (May *et al*., 1998; Noctor and Foyer, 1998; Xiang and Oliver, 1998; Foyer and Noctor, 2003; Freeman *et al*., 2005). Indeed, GSH acts as an antioxidant, quenching the reactive oxygen species (ROS) generated in response to stress before ROS cause damage to cells (Navari-Izzo *et al*., 1997). Glutathione serves an additional function in plant responses to heavy metal stress as a precursor of PCs (Rauser, 1995; Cobbett and Goldsbrough, 2002). PCs are synthesized from GSH by the enzyme phytochelatin synthase (Cobbett, 2000) and comprise a family of intracellular heavy metal binding peptides with the same basic structure [(γ-L-Glutamyl-L-Cysteinyl)$_{2–11}$-glycine] (Cobbett *et al*., 1998; Nocito *et al*., 2006). The formation of PCs is induced following the exposure of plants to a number of metal(loid)s such as Cd and As (Clemens *et al*., 1999; Schmöger, Oven, and Grill, 2000; Hartley-Whitaker *et al*., 2001; Heiss *et al*., 2003). In support of the involvement of PCs in Cd tolerance, *Ar abidopsis cad1* mutants were shown to be Cd-sensitive because of their lack in GSH and PCs (Howden *et al*., 1995). Interestingly, the overexpression of phytochelatin synthase in *Arabidopsis* enhanced As tolerance, but led to Cd hypersensitivity (Lee *et al*., 2003), perhaps due to GSH depletion. *In-planta* x-ray absorption spectroscopy studies confirmed the complexation of Cd—as well as As—to S ligands, presumably the thiols of GSH and PCs (Pickering *et al*., 1999, 2000). In our previous studies, two *Escherichia coli* enzymes involved in GSH biosynthesis, GS or γ-ECS, were constitutively expressed in *Brassica juncea* (Zhu *et al*., 1999a, 1999b). The expression of either GS or γ-ECS in this plant species resulted in higher accumulation of GSH and PCs, as well as in enhanced Cd tolerance and accumulation.

In the current study, the transgenic GS and glutamylcysteine synthetase (ECS) *B. juncea* plants were further examined for their responses to other heavy metals in order

to evaluate the ability of GS and ECS transgenics to tolerate and accumulate a number of metals and combinations of metals. If the transgenics show an enhanced capacity to tolerate and accumulate other elements besides Cd, this may broaden their applicability in phytoremediation technologies. Promising in this respect is that the ECS and GS plants have already shown enhanced Cd and Zn accumulation from metal-polluted soil collected in Leadville, CO (Bennett *et al*., 2003), as well as improved Se accumulation *in situ* from Se-polluted soil in the San Joaquin Valley, CA (Banuelos *et al*., 2005).

MATERIALS AND METHODS

Plant Material

Brassica juncea (Indian mustard) wild-type (WT) seeds were from the accession no. 173874, North Central Regional Plant Introduction Station (Ames, IA). Transgenic GS and ECS plants were obtained through transformation of plants from this same accession number using GS and $\gamma-$ECS gene constructs containing the *Escherichia coli gshII* and *gshI* genes, respectively. The gene constructs used in this study were described previously (Strohm *et al*., 1995; Noctor *et al*., 1998). The double-enhanced 35S cauliflower mosaic virus promoter drove both the *gshII* and *gshI* genes, and the kanamycin-resistance gene (*nptII*) under control of the nopaline synthase promoter was used as a marker gene. The bacterial GS and ECS enzymes lacked any characterised localization targeting signals and, therefore, were predicted to be localized to the cytosol. Hypocotyls of *B. juncea* were transformed using *Agrobacterium tumefaciens*–mediated transformation as described in Pilon-Smits *et al*. (1999). Molecular characterization of the transgenic lines was performed by PCR and Western blotting, as described (Zhu *et al*., 1999a, 1999b), and the GS and ECS transgenics were shown to express the bacterial proteins in question.

For this study we tested two independently obtained, homozygous plant lines for each type of transgenic: cytGS2, cytGS7, cytECS3 and cytECS8. Wild-type *B. juncea* was included as a control.

Metal Tolerance and Accumulation Experiments

Seedling experiments. Seeds from WT *B. juncea* and from transgenic lines GS2, GS7, ECS3, and ECS8 were surface sterilized by rinsing in 95% ethanol for 1 minute, then in 20% sodium hypochlorite (NaClO) for 30 minutes, while rocking on a platform. Sterilized seeds were then washed with sterile distilled water for 5 $\times$ 10 minutes and sown in sterile Magenta boxes (Sigma, St. Louis, MO) with a density of 30 seeds per box. Each experiment was performed in parallel with its own control (*i.e*., using growth medium lacking the additional metal or metalloid). The agar medium in the magenta boxes contained 0.5 strength Murashige and Skoog (1962) (MS) salts and vitamins (M5524; Sigma, St Louis, MO), including 1% (p/v) sucrose and 0.4% (p/v) agar, and was supplemented with the following concentrations of metal(loid)s: 5 ppm Cu, 40 ppm Zn, 85 ppm Mo, 75 ppm Mn, 75 ppm Pb, 4 ppm As, 10 ppm Ni, or 10 ppm Cr, in the form of $CdCl_2$, $Pb(NO_3)_2$, $MnSO_4$, $NaAsO_2$, K_2CrO_4, $CuSO_4$, Na_2MoO_4, and $ZnCl_2$, respectively. Mixed-metal experiments were also performed, where either 1.5 ppm As, 7.5 ppm Cd, and 12 ppm Zn or 1.5 ppm As, 7.5 ppm Cd, and 25 ppm Pb were added to the media. The chemical forms were chosen to have high solubility in water and to have a minimal effect on the nutrient solution composition. No precipitation was observed when these metal salts were added to agar

medium or nutrient solution. Before performing the experiments with the transgenic lines, we determined which concentration of each metal(loid) was optimal for our experiments, *i.e.*, the concentration giving a 50% reduction in WT seedling fresh weight, corresponding with an approximately 75% reduction in seedling root length as compared to untreated controls.

The seeds were allowed to germinate in the cold room for 2 days and then germinated seedlings were transferred to a growth chamber for 7 days at 24°C and a 16-hour photoperiod, under a photon flux density of 40 mmol m^{-2} s^{-1}. Seedlings were then harvested and carefully washed with distilled water. The root length and fresh weight of seedlings were measured and the tolerance index (TI) was calculated as the growth on metal-containing medium divided by the growth on control medium. The root length tolerance index (RLTI) and fresh weight tolerance index (FWTI) are commonly used to quantify plant metal tolerance (Murphy and Taiz, 1995). Seedlings from each treatment were frozen in liquid nitrogen and stored at −80°C for thiol analysis. The remaining seedlings were dried and prepared for elemental analysis on the total seedlings.

Mature plant experiments. WT and transgenic mature plants were compared for their ability to tolerate and accumulate metals in a hydroponics setup. The experiments were performed using selected metals to which the transgenic lines were more tolerant than WT at the seedling level. Experiments for GS and ECS overexpressing lines were performed separately. Seeds of WT and GS and ECS transgenics were surfacesterilized, sown on agar medium as described earlier, and grown in magenta boxes for 4 days. Subsequently, the seedlings were transferred to a hydroponics setup containing a continuously aerated 0.5 strength Hoagland's nutrient solution (Hoagland and Arnon, 1938) and were allowed to grow in a greenhouse at 22°C and a 16-hour photoperiod. The nutrient solution was replaced daily. After 2 weeks, the plants were transferred to 21.5-L plastic containers at a density of 12 plants per box, which were aerated through fish tank pumps. The fresh weights of the plants were measured and, after a 24-hour period of adjustment to the new medium, the individual metal treatments were started. Metal(loid)s were added to the 0.5 Hoagland's nutrient solution to a final concentration of 7.5 ppm Cd, 5 ppm As, 5 ppm Cr, or 40 ppm Mo for the experiment in which GS transgenics were tested. In the experiments using ECS plants, the metal(loid) concentrations were 2.5 ppm As, 7.5 ppm Cd, and 60 ppm Mo. The metals were supplied in the form of $NaAsO_2$, $CdCl_2$, K_2CrO_4, and Na_2MoO_4, respectively. The concentration of metals used in these experiments was chosen because they were shown earlier to result in approximately 50% FW reduction in WT plants (Lindblom *et al.*, 2006) and/or on the basis of metal levels reported for polluted areas (Ross, 1994; Cappuyns *et al.*, 2002). The treatment solutions were replaced every 4 days. After 14 days of treatment, each plant was washed under running water to remove any trace metals adhering to the root surface. The plant fresh weight was measured, and samples from the roots and shoots were frozen in liquid nitrogen for analysis of thiol levels. The plants were dried for 3 days at 70°C and dry weights were recorded. The dry roots and shoots were ground and prepared for elemental analysis as described below.

Elemental Analysis

Pooled samples of entire seedlings (five samples pooled from 14 plants each), or shoot and root samples from mature plants (12 per treatment), were digested with nitric acid according to the method of Zarcinas, Cartwright, and Spouncer (1987). The total elemental concentration of As, Cd, Cr, and Mo in the digests was measured *via* inductively

coupled plasma atomic emission spectroscopy (ICP-AES, Thermo Jarrell Ash, Franklin, MA) according to the method of Fassel (1978), using appropriate standards (National Institute of Standard and Technology). Plants untreated with any metal(loid)s were analyzed for metal content as negative controls. The values obtained were expressed in mg element kg^{-1} d. w.

Quantification of Thiol Content

Thiols, including GSH and PCs, were measured by post-column derivatization with the thiol reactive dye monobromobimane after separation *via* reverse-phase HPLC (high performance liquid chromatography). Frozen foliar and root tissues of *B. juncea* plants were plunged into 2 mL of 10 mM methanesulfonic acid at 70°C for 2 minutes and subsequently homogenized with a mortar and pestle. The resulting solutions were centrifuged at 9000 rpm for 10 minutes at 4°C and the supernatants were retained for further analysis. Thiol derivatization was performed by first adding borate-diethylenetriaminepentaacetic acid (borate-DTPA; 100 mM and 10 mM, respectively) buffer (pH 9) and tris-2-carboxyethyliphosphin hydrochloride (TCEP) to 0.2 mL of the sample and then incubating it for 10 minutes at room temperature. Then monobromobimane (50 mM in acetonitrile) was added to the samples undergoing incubation for 30 minutes at 45°C. Subsequently acetic acid was added following incubation of the samples with TCEP for 5 minutes at room temperature. Resulting monobromobimane derivatives were detected fluorometrically (Waters 474 Scanning fluorescence detector) at 470 nm by excitation at 380 nm after separation by reverse-phase HPLC using a Waters HPLC System (Waters Multisolvent Delivery System, Autosampler 717 plus) connected to a Waters Xterra C8 2.1 × 250 mm column. Thiols were separated by applying a flow rate of 0.2 mL/min and using a gradient of acetate buffer (0.25% acetic acid, 8% acetonitrile, pH 4, 0.1 mM tetrahexylammonium bromide), and 100% acetonitrile. Glutathione, glutamyl-cysteine, and cysteine standards were obtained from Sigma, while phytochelatin standards were furnished by the UC Berkeley Microchemical Facility.

Statistical Analysis

The software program JMP-IN (SAS Institute, Cary, NC, 2005) was used for the statistical analysis of metal tolerance and accumulation data. Analysis of variance (ANOVA) was performed followed by pair-wise post-hoc analyses to determine which means differed significantly ($\alpha = 0.05$). Statistically significant differences ($p < 0.05$) are reported in the text and shown in the figures and tables.

RESULTS

Metal Tolerance at the Seedling Level

In order to compare the metal tolerance of transgenic *B. juncea* overexpressing γ-glutamylcysteine synthetase (ECS) or GS relative to WT plants, the tolerance of these plant types to a variety of metal(loid)s was evaluated at the seedling level and judged from the growth parameters' root length and fresh weight.

The GS and ECS seedlings could tolerate a number of metals better than WT (Figure 1). When treated with Cr, Mn, or Mo, the ECS3 and ECS8 lines both showed higher root length tolerance index (RLTI) and, thus, less root growth inhibition than WT

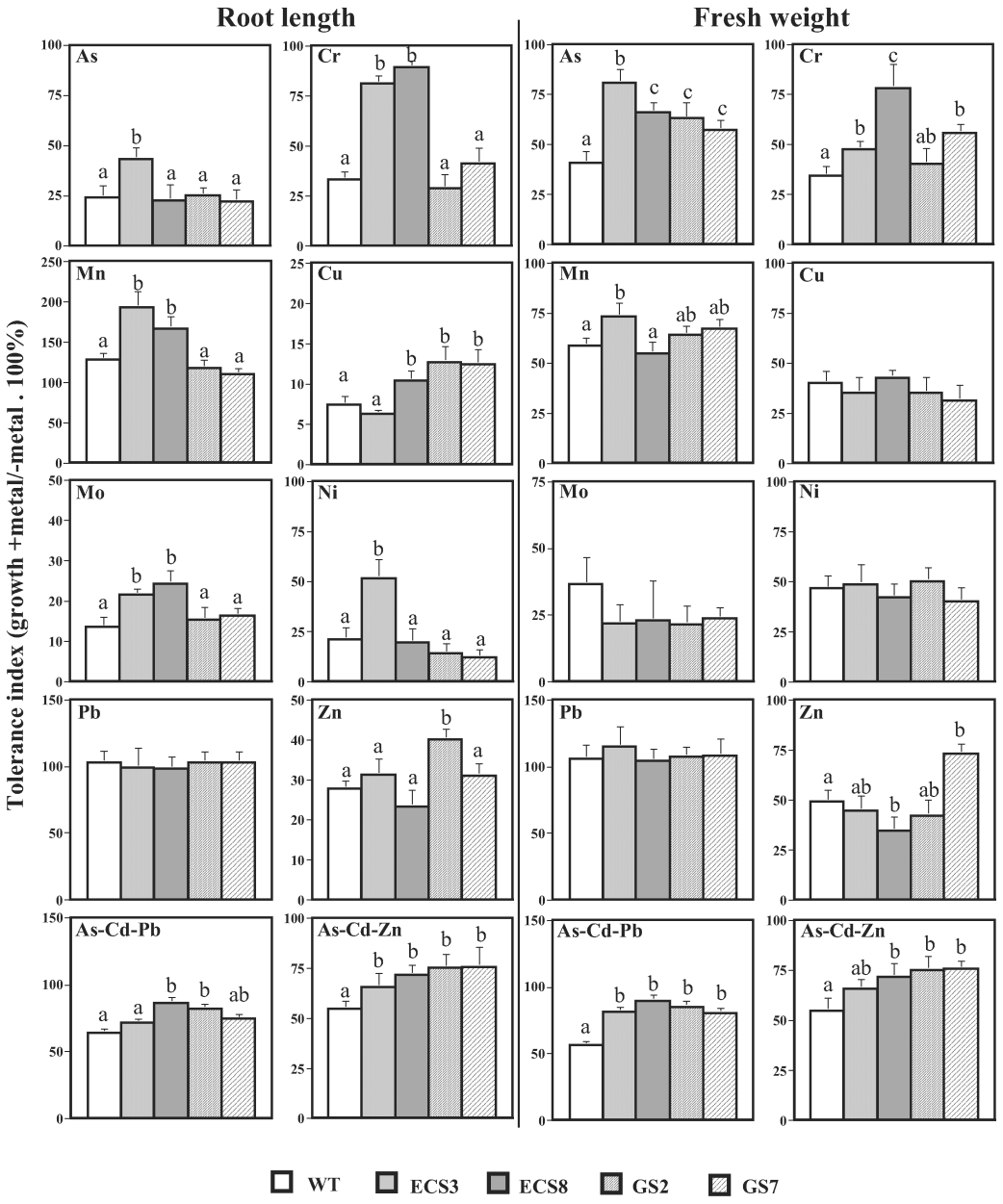

Figure 1 Seedling metal tolerance for WT *B. juncea* plants and transgenics overexpressing ECS (ECS3, ECS8) or GS (GS2, GS7). Shown on the left is the ratio of seedling root length grown on medium containing metal(loid) relative to root length on control medium. Shown on the right is the ratio of seedling fresh weight grown on medium containing metal(loid) relative to root length on control medium. The form and concentration used for each metal(loid) is described in the Materials and Methods section. Shown are means ± SE ($n = 30$). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

(Figure 1). In addition, ECS3 seedlings were more tolerant to As and Ni, and ECS8 seedlings were slightly more Cu tolerant (Figure 1). Relative to WT, GS lines 2 and 7 showed greater tolerance to Cu and the GS2 transgenics had longer roots under Zn treatment (Figure 1). The treatment of seedlings with the mixed metal combination of As, Cd, and Zn resulted in an

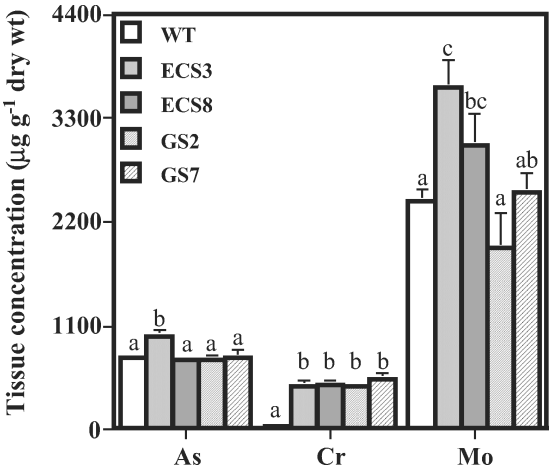

Figure 2 Seedling metal accumulation for WT *B. juncea* plants and transgenics overexpressing ECS (ECS3, ECS8) or GS (GS2, GS7). Shown is the tissue metal concentration after 7 days of growth on agar medium supplied with a metal(loid) to a concentration as indicated in the Materials and Methods section. Shown are means ± SE ($n = 5$ samples pooled from 14 plants each). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

overall increase in root length of all four transgenic lines compared to WT. The ECS8 and GS2 transgenics were a little more resistant than WT to the As, Cd, and Pb combination.

With respect to a plant's metal tolerance calculated on the basis of fresh weight, all four transgenic lines were more tolerant to As than WT (Figure 1). The ECS3 and ECS8 lines also had improved tolerance to Cr as compared to WT. The GS7 transgenics were more tolerant to Cr and Zn, and ECS3 plants were more tolerant to Mn but less tolerant to Zn. For the two mixed-metal treatments of As, Cd, and either Pb or Zn, the fresh weight tolerance index of ECS8 and GS2 and GS7 transgenics was greater than WT; ECS3 showed the same pattern of tolerance although they were not significant for As–Cd–Pb and As–Cd–Zn mixed-metal combination as measured by root length and fresh weight, respectively (Figure 1).

Seedling Metal Accumulation

In followup seedling experiments using three selected metals, the tissue concentration of As, Cr, and Mo in WT, GS, and ECS *B. juncea* seedlings was measured. Relative to WT, the ECS3 and ECS8 seedlings contained significantly higher levels of Cr and Mo, and ECS3 also contained more As (Figure 2). Both GS lines accumulated higher concentrations of Cr than WT, but no significant differences in As or Mo accumulation between GS transgenics and WT were observed (Figure 2).

GSH, PC2, PC3, and PC4 Levels in Seedlings

The concentration of these thiols was determined in seedlings treated with or without As, to obtain an indication of the effect of overexpression of the GSH-synthesizing enzymes ECS and GS on tissue GSH and phytochelatin (PC) levels. When treated with As, the ECS and GS seedlings contained more GSH (except for GS7), PC2, PC3, and PC4 (except for GS2) than WT (Figure 3 A–D). The accumulation of GSH, PC2, and PC4 was particularly

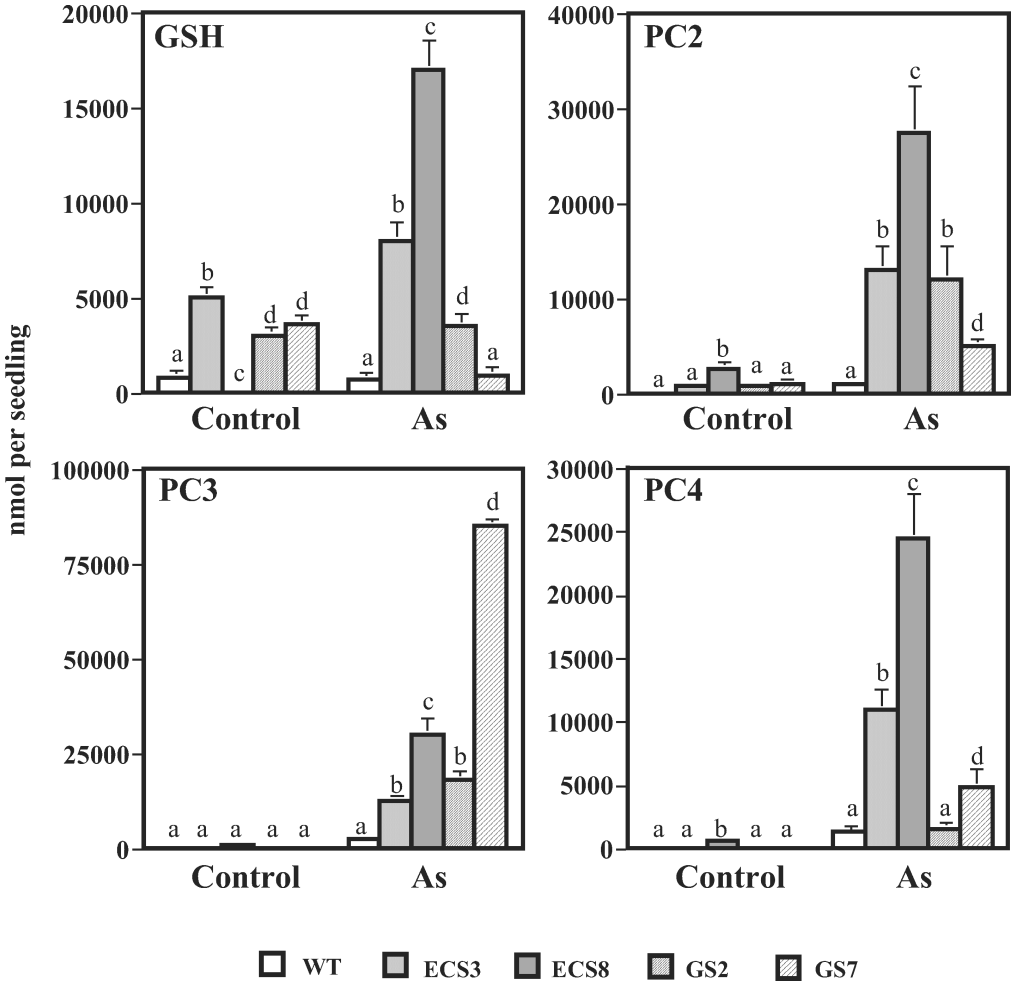

Figure 3 Tissue concentrations of GSH, phytochelatins (PC2, PC3, PC4), in WT *B. juncea* plants and transgenics overexpressing either ECS (ECS3, ECS8) or GS (GS2, GS7). *B. juncea* seedlings were grown in the absence or presence of 4 ppm $NaAsO_2$. Note that tissue PC3 and PC4 levels in the absence of As were below the detection limit in all of the samples except PC4 in ECS8. Shown are means ± SE ($n = 3$ samples pooled from four plants each). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

pronounced in ECS8, which contained up to 30-fold higher levels than WT. Under control conditions, there was more GSH in the transgenic lines relative to WT, except for ECS8. While ECS8 showed no measurable GSH under control conditions, it had the highest level of PC2 (Figure 3 A and B). Under control conditions, the values of PC3 and PC4 were below the detection limit, except for ECS8, where low levels of both peptides were detected (Figure 3 C and D).

Metal Tolerance at the Mature Plant Level

The tolerance experiments were performed in mature *B. juncea* plants growing in a hydroponic system and treated for 14 days with a selected number of metals, specifically those metals that the transgenic seedlings either tolerated or accumulated better than WT.

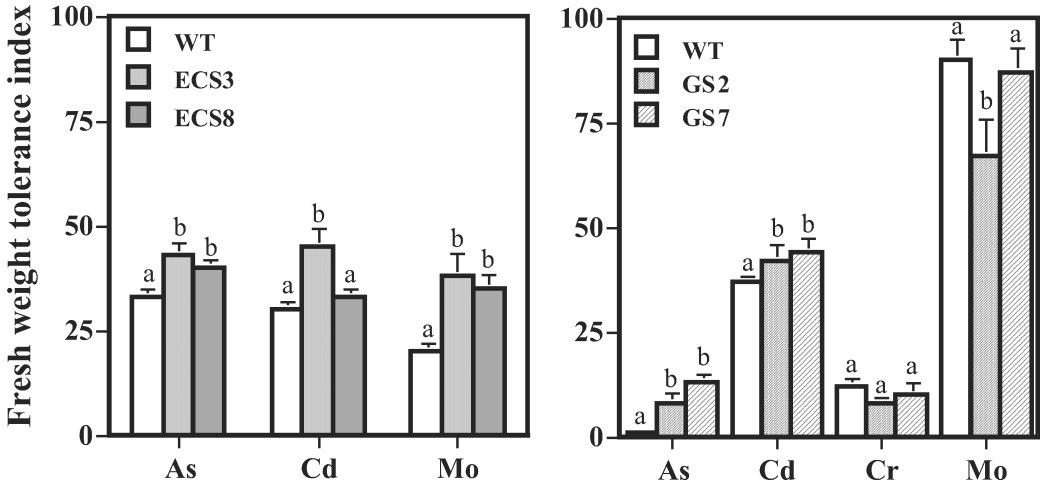

Figure 4 Mature plant metal tolerance for WT *B. juncea* plants and transgenics overexpressing ECS (ECS3, ECS8) or GS (GS2, GS7). Shown is the ratio of plant fresh weight when grown on medium containing metal(loid) relative to its fresh weight on control medium. The form and concentration used for each metal(loid) is described in the Materials and Methods section. Shown are means ± SE ($n = 10$). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

GS plants were treated with either As, Cd, Cr, or Mo, whereas ECS plants were provided with either As, Cd, or Mo (Figure 4). In parallel, the same plant types were grown in a medium without added metals to serve as the control. Moreover, for each experiment using transgenics, WT plants were grown in parallel for comparison. Glutathione synthetase plants tolerated Cd and As significantly better than WT (Figure 4). The FWTI of GS2 and GS7 plants were 20%–25% and 50%–80% higher than WT when treated with Cd or As, respectively. No enhanced tolerance was observed for Cr or Mo; in fact, GS2 plant growth was lower than that of WT on Mo. In the ECS mature plant experiment, the FWTI was used to compare WT and transgenic lines because the mean values under control growth conditions were significantly different between plant lines. The tolerance indices for ECS3 and ECS8 were higher than WT for As and Mo, and ECS3 also showed improved growth on Cd (Figure 4).

Mature Plant Metal Accumulation

The tissue concentration of the treatment metals was also measured in transgenic mature plants and by multiplying the tissue concentration with root or shoot dry weight, the total metal accumulation per plant organ was calculated. The Mo concentration in the shoot of Mo-treated ECS3 plants was 38% higher than in WT plants (Figure 5A) and, after factoring in shoot dry weight, ECS plants accumulated 47% more total Mo in their shoot than WT (Figure 5B). In the roots, ECS3 plants also contained a slightly higher Mo concentration than WT (Figure 5C; NS) and a higher total amount of Mo (Figure 5D). Line ECS8 did not show a similar pattern for Mo as ECS3. The shoot Cd concentration was not significantly different from WT in the ECS lines (Figure 5A) and ECS8 accumulated less total Cd in its shoot than WT due to a lower shoot weight (Figure 5B). In roots, the Cd concentration was 27% and 40% higher than WT in ECS3 and ECS8, respectively (Figure 5C, $p < 0.05$ for ECS8 only). However, no significant differences were recorded between ECS transgenics and

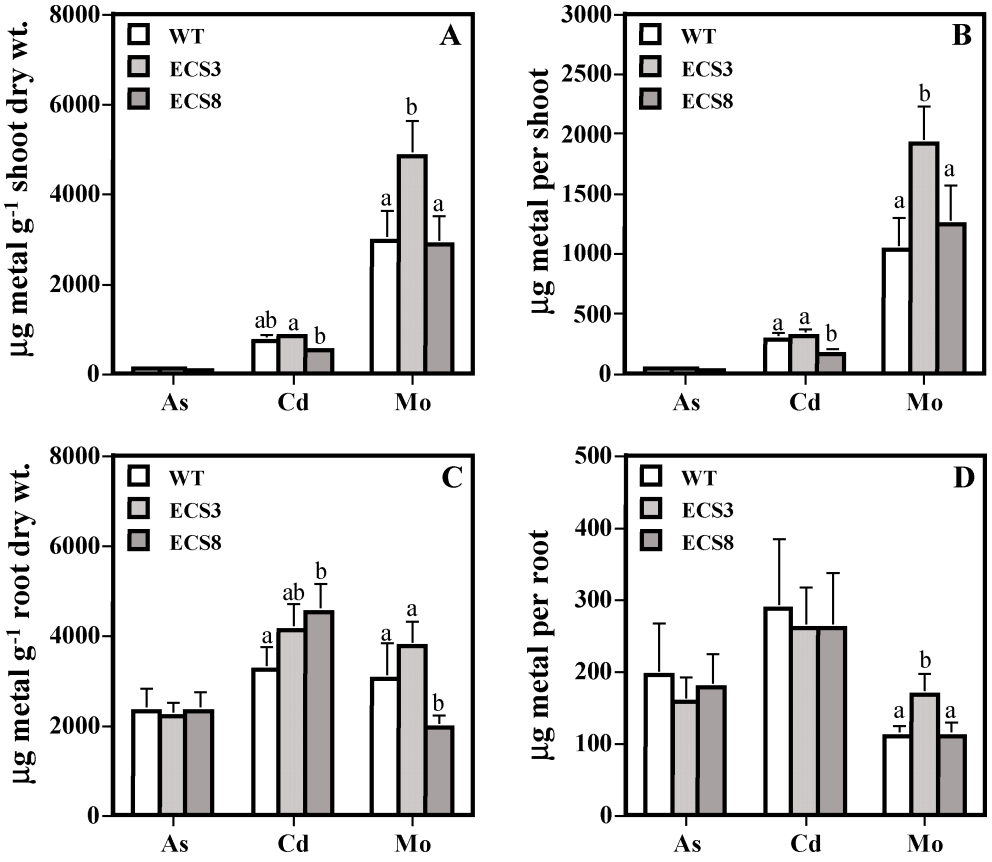

Figure 5 Mature plant metal accumulation for WT and ECS overexpressing (ECS3, ECS8) *B. juncea* plants. Shown is the shoot and root metal concentration and total metal accumulation after hydroponically grown plants were treated for 14 days with a metal(loid) concentration as indicated in the Materials and Methods section. Shown are means ± SE ($n = 10$). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

WT for total root Cd accumulation, due to lower root biomass (Figure 5D). The ECS lines did not contain more As compared to WT (Figure 5A–D).

The GS transgenics had a higher tissue Cd concentration and larger total Cd accumulation in their shoot (Figure 6A and B). In the root they also accumulated more total Cd (Figure 6D, $p < 0.05$ only for GS7). The GS7 transgenics did not show any differences in shoot As or Cr concentration relative to WT (Figure 6A); they accumulated 30% more total As and Cr than WT per shoot, but these were not statistically significant differences (Figure 6B). In the root, the only significant difference in metal concentration was observed for GS7 on Cr (Figure 6C); root Cd levels were slightly higher in both GS lines as well, but this was not significant. On a per root basis, however, both GS2 and GS7 accumulated significantly more Cr than WT and GS7 also accumulated more Cd (Figure 6D); GS2 showed elevated Cd accumulation as well, but this was not significant. No differences in Mo shoot or root accumulation were observed.

There were no significant differences in the root or shoot concentration of Ca, Cu, Fe, Mg, Mn, or S between the transgenic lines and WT for any treatment (data not shown).

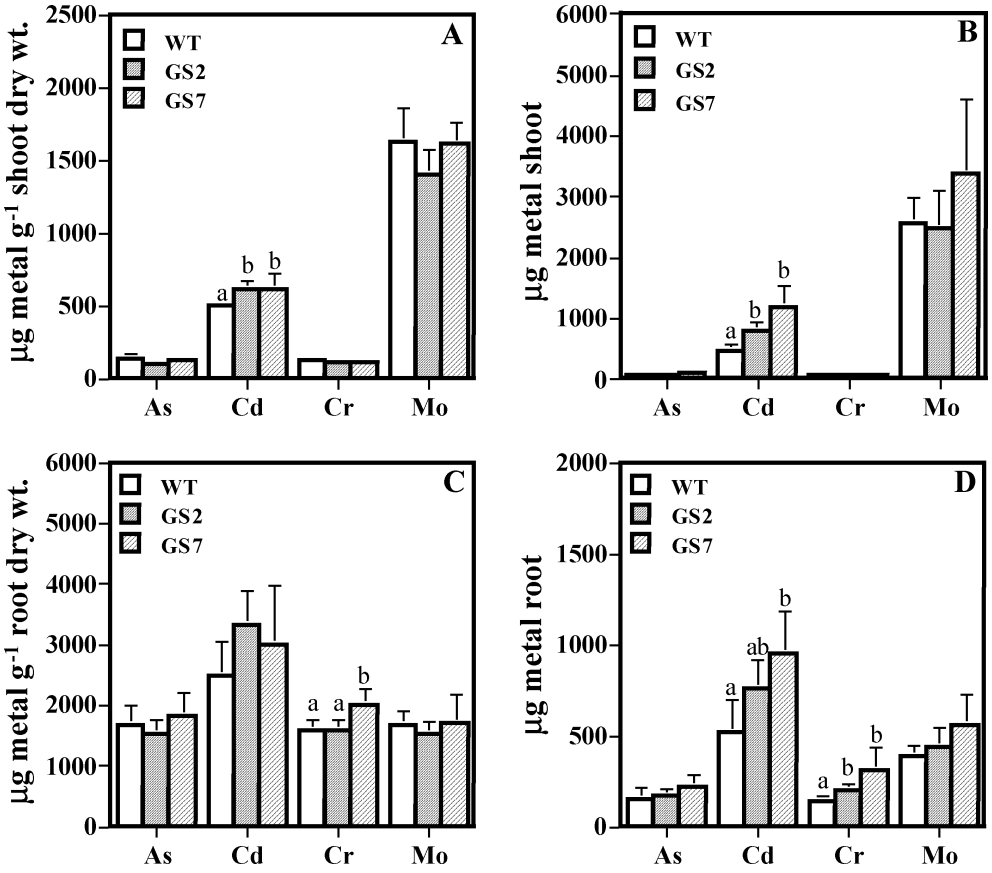

Figure 6 Mature plant metal accumulation for WT and GS overexpressing (GS2, GS7) *B. juncea* plants. Shown is the shoot and root metal concentration and total metal accumulation after hydroponically grown plants were treated for 14 days with a metal(loid) concentration as indicated in the Materials and Methods section. Shown are means ± SE ($n = 10$). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

DISCUSSION

The results presented here indicate that, compared to WT plants, the ECS and GS transgenics have enhanced the capacity to tolerate and accumulate several metal/loids. On the basis of root length, the ECS seedlings showed enhanced tolerance to Cr, Mn, or Mo while the GS lines were more tolerant to Cu or Zn than WT. On the basis of fresh weight, both ECS and GS seedlings were more tolerant to As. In the mature plant studies, which were performed using selected metals, *i.e.*, As, Cd, or Mo for ECS lines and As, Cd, Cr, or Mo for GS transgenics, GS plants showed better growth than WT when treated with As, but did not exhibit enhanced tolerance to Cr or Mo. ECS mature plants showed increased tolerance to As and Mo. Together with our earlier studies (Zhu *et al.*, 1999a, 1999b), which showed that ECS and GS plants are more tolerant to Cd, these findings provide evidence that GSH and PCs are part of a general metal detoxification mechanism in *B. juncea*. The observed increased levels of GSH and PCs found in transgenics when treated with As (Figure 3) or Cd (Zhu *et al.*, 1999a, 1999b) are presumably responsible for their enhanced tolerance to these metals, because both GSH and PCs are able to chelate metals and facilitate their sequestration in the vacuole. Also in support of this hypothesis are results recently obtained

by overexpression of PC synthase in *B. juncea* plants, where the increased tolerance to As, Cd, or Zn was accompanied by higher accumulation of GSH and PCs (Gasic and Korban, 2006, 2007). The overexpression of γ-ECS or GS or their co-expression in *Arabidopsis thaliana* was further shown by Li *et al*. (2006) to enhance the tolerance to mercury and As, and resulted in higher levels of GSH, PC2, PC3, and PC4 in roots compared to WT plants. Despite these studies emphasizing the positive role of high levels of PCs in plant detoxification, Lee *et al*. (2003) found that PC synthase overexpression in *A. thaliana* resulted in Cd hypersensitivity. The authors ascribed this to the toxicity exerted by PCs present at supraoptimal levels and by GSH depletion. However, in a more recent study, Kim and Lee (2007)suggest that Cd hypersensitivity in PC synthase overexpressing transgenics may be due to the overexpressed PC synthase itself, since enzyme can bind metals.

In some cases, different results with respect to metal tolerance were observed at the seedling and mature life stages. These differences may be due to the different concentration of metal/loid used for the experiments and/or to differences in metabolic activities between these life stages, which may be associated with different enzyme limitations for metal tolerance. Also, the GS and ECS transgenics did not show enhanced tolerance to the same set of metals in all cases, even though they are part of the same biochemical pathway. It is possible that under different stress conditions the production of GSH and PCs is limited by ECS or by GS, respectively. Possibly, the downstream enzyme PC synthase was limiting in cases where the ECS and GS overexpressors showed no difference in metal tolerance compared to WT. However, when tested, the PC levels were enhanced in the transgenics when ECS or GS were overexpressed, indicating that PC synthase was not rate-limiting for PC production.

In addition to being more metal-tolerant, plants overexpressing bacterial GS or ECS accumulated appreciably more of several metals than WT. ECS seedlings accumulated significantly higher levels of Mo than WT and the concentration of As in ECS3 also was significantly higher than in WT. In addition, seedlings from all four ECS or GS lines contained more Cr relative to WT. Adult GS and ECS plants showed increased concentration of all metals tested except As, as compared to WT. Previous data published by this laboratory also showed increased Cd accumulation by GS and ECS overexpressing lines (Zhu *et al*., 1999a, 1999b). The increase in total metal accumulation observed in these transgenics, as calculated from the metal concentration multiplied with dry weight, may be because, over time, the improved metal tolerance due to GS and ECS overexpression allowed the plants to produce more biomass and, therefore, accumulate more of the various metals than WT.

The increased tolerance to combinations of metals at the tested metal concentrations by GS and ECS overexpressing *B. juncea* represents a promising result for future practical applications in phytoremediation, as combinations of metals are very common on polluted sites, to levels similar to the ones used in these studies (Cappuyns *et al*., 2002). Indeed, these same ECS and GS transgenics showed enhanced accumulation of several heavy metals from polluted soil collected at a heavily polluted site near Leadville, CO (Bennett *et al*., 2003), in a greenhouse experiment. Furthermore, since many sites are polluted with mixtures of metals and organics, it is encouraging that the ECS and GS plants also showed enhanced tolerance to certain organic pollutants (Flocco, Lindblom, and Pilon-Smits, 2004), perhaps by providing more GSH for conjugation, facilitating vacuolar sequestration. Additional experiments examining the tolerance of mature ECS and GS plants on soils containing combinations of metals or metals and organics should provide more information about the extent of the GS and ECS transgenic plants' ability to tolerate and remediate multiple pollutants simultaneously.

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