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Cadmium Tolerance and Accumulation in Indian Mustard Is Enhanced by Overexpressing γ-Glutamylcysteine Synthetase[1]

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To investigate rate-limiting factors for glutathione and phytochelatin (PC) production and the importance of these compounds for heavy metal tolerance, Indian mustard (*Brassica juncea*) was genetically engineered to overexpress the *Escherichia coli gshI* gene encoding γ-glutamylcysteine synthetase (γ-ECS), targeted to the plastids. The γ-ECS transgenic seedlings showed increased tolerance to Cd and had higher concentrations of PCs, γ-GluCys, glutathione, and total non-protein thiols compared with wild-type (WT) seedlings. When tested in a hydroponic system, γ-ECS mature plants accumulated more Cd than WT plants: shoot Cd concentrations were 40% to 90% higher. In spite of their higher tissue Cd concentration, the γ-ECS plants grew better in the presence of Cd than WT. We conclude that overexpression of γ-ECS increases biosynthesis of glutathione and PCs, which in turn enhances Cd tolerance and accumulation. Thus, overexpression of γ-ECS appears to be a promising strategy for the production of plants with superior heavy metal phytoremediation capacity.

Heavy metals and metalloids such as Cd, Pb, Hg, As, and Se are an increasing environmental problem worldwide. Plants can be used to remove heavy metals by accumulating, stabilizing, or biochemically transforming them. This cost-effective and environment-friendly technology has been called "phytoremediation" (Salt et al., 1995). Hyperaccumulators—plant species that accumulate extremely high concentrations of heavy metals in shoots—offer one option for the phytoremediation of metal-contaminated sites. However, hyperaccumulators tend to grow slower and produce little biomass (Brooks, 1994). An alternative approach is to genetically engineer fast-growing species to improve their metal tolerance and metal-accumulating capacity. A suitable target species for this strategy is Indian mustard (*Brassica juncea*), which has a large biomass production, a relatively high trace element accumulation capacity (Dushenkov et al., 1995), and can be genetically engineered (Zhu et al., 1999).

Non-protein thiols (NPTs), which contain a high percentage of Cys sulfhydryl residues in plants, play a pivotal role in heavy metal detoxification. The reduced form of glutathione (γ-Glu-Cys-Gly, GSH) is one of the most important components of NPT metabolism. GSH may play several roles in heavy metal tolerance and sequestration. It protects cells from oxidative stress damage, such as that caused by heavy metals in plants (Gallego et al., 1996; Weckx and Clijsters 1996, 1997). For example, it has been shown that Cd treatment causes an increase in lipid peroxidation and lipooxygenase activity (Gallego et al., 1996). GSH is the direct precursor of phytochelatins (PCs). PCs are heavy metal-binding peptides involved in heavy metal tolerance and sequestration (Steffens, 1990). PCs comprise a family of peptides with the general structure $(\gamma\text{-Glu-Cys})_n$-Gly, where $n = 2$ to 11 (Rauser, 1995). They were shown to be induced by heavy metals such as Cd in all plants tested (Zenk, 1996), including Indian mustard (Speiser et al., 1992). The roles of GSH and PC synthesis in heavy metal tolerance were well-illustrated in Cd-sensitive mutants of Arabidopsis. For example, the Cd-sensitive *cad2* mutant was defective in GSH and PC biosynthesis (Howden et al., 1995).

GSH is synthesized from its constituent amino acids in two sequential, ATP-dependent enzymatic reactions, catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS), respectively. PC synthase subsequently catalyzes the elongation of the $(\gamma\text{-Glu-Cys})_n$ by transferring a γ-GluCys group to GSH or to PCs (Zenk, 1996). Genes encoding PC synthase have just recently been cloned from plants and yeast (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999). The rate-limiting step for GSH synthesis in the absence of heavy metals is believed to be the reaction catalyzed by γ-ECS, since the activity of this enzyme is feedback-regulated by GSH and dependent on Cys availability (Noctor et al., 1998b). This view was supported by the observation that overexpression of the *Escherichia coli gshI* gene (which encodes γ-ECS) in poplar resulted in increased foliar GSH levels (Arisi et al., 1997; Noctor et al., 1998a). In contrast, overexpression of GS did not lead to an increase in GSH levels in poplar (Foyer et al., 1995) or in Indian mustard (Zhu et al., 1999) in the absence of heavy metals. However, the Indian mustard GS-overexpressing plants showed higher levels of GSH and

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PC2 relative to untransformed plants in the presence of heavy metals. These GS plants also showed enhanced heavy metal tolerance and accumulation (Zhu et al., 1999).

Although γ-ECS plays an important role in controlling GSH synthesis, its significance in controlling PC synthesis and heavy metal tolerance or accumulation remains unclear. It has been reported that overexpression of tomato γ-ECS could restore some degree of heavy metal tolerance to the *cad2* Arabidopsis mutant. However, overexpression of this gene did not increase the Cd tolerance of wild-type (WT) Arabidopsis plants (Goldsbrough, 1998). In the present study we overexpressed the *E. coli* γ-ECS enzyme in the chloroplasts of Indian mustard. The transgenic γ-ECS plants were compared with WT Indian mustard plants with respect to their Cd accumulation and tolerance, as well as their levels of heavy metal binding peptides.

MATERIALS AND METHODS

Plant Transformation and Characterization

Indian mustard (*Brassica juncea*, accession no. 173874) seeds were obtained from the North Central Regional Plant Introduction Station (Ames, IA). Hypocotyl segments from 3-d-old axenically grown seedlings were transformed as described by Pilon-Smits et al. (1999). The γ-ECS gene construct used was described earlier by Noctor et al. (1998a). It contains 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 (P70). The construct also contains the *nptII* gene, which confers kanamycin resistance. This construct was shown earlier to successfully target the *E. coli* γ-ECS protein to poplar plastids (Noctor et al., 1998a).

PCR was used to identify γ-ECS transgenic lines among the kanamycin-resistant lines obtained. The PCR primers used were the following: the forward primer was directed against the 35S promoter, with the sequence 5′-CCT TCG CAA GAC CCT TCC TC-3′. The reverse primer was directed at the *gshI* gene and had the sequence 5′-GCA CTC GGT TTT CTC AAA CGG-3′.

Total RNA was isolated from 7-d-old seedling shoots using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Northern-blot hybridization was carried out as described by Krumlauf (1994) using a *gshI* DNA probe that was generated by PCR using the primers described above. The template DNA was a purified plasmid containing the *gshI* gene. The PCR product was purified from the gel and labeled with ^{32}P-[dCTP] by random priming using a DNA labeling kit (Ready-To-Go, Pharmacia Biotech, Piscataway, NJ).

For western blotting, 7-d-old seedlings (shoots and roots separately) were ground in liquid nitrogen and extracted in 50 mM potassium phosphate buffer, pH 8.0, added at 1 mL g^{-1} fresh weight. After measurement of the total protein concentration (Bradford, 1976), 10 μg of protein from each sample was separated by SDS-PAGE and blotted onto a Zeta-probe membrane (Bio-Rad, Hercules, CA) by electroblotting. We used the Bio-Rad Immuno-lite kit for the immunodetection of separated proteins, and rabbit serum raised against purified *E. coli* γ-ECS as the first antibody (Arisi et al., 1997).

Plant Growth and Tolerance Experiments

Seedling Experiments

Two similar seedling experiments (A and B) were conducted and described here. In experiment A, T2 seeds from transgenic lines ECS2, ECS4, and WT Indian mustard were sterilized by rinsing in 95% ethanol for 30 s, then in 1% hypochlorite solution for 30 min, and subsequently in sterile deionized water five times for 10 min each time, all on a rocking platform. Fifty sterilized seeds were sown in a grid pattern in Magenta boxes (Sigma, St. Louis) on half-strength Murashige and Skoog medium containing 10 g L^{-1} Suc, 5 g L^{-1} Phytagar (Sigma) and different concentrations of $CdSO_4$ (0, 0.15, 0.20, or 0.25 mM). After 7 d at 25°C under continuous light, the individual seedlings were harvested, washed, and weighed, and the length of the longest root was measured. Total NPT concentration and total glutathione were also measured from this experiment. Seedling experiment B was essentially the same as experiment A except for the following: the treatments were 0 and 0.2 mM Cd, the medium contained 4 g L^{-1} Phytagar, and Cd-treated seedlings were harvested after 11 d. This experiment focused on individual thiol measurements. Root length was also measured but data are not presented since the results were similar to those from experiment A.

Mature Plant Experiments

Seeds of ECS2, ECS4, and WT Indian mustard were sterilized and sown in Magenta boxes as described above. After 5 d on agar, the seedlings were transferred to 4-inch pots containing coarse sand. The pots were maintained in a greenhouse with controlled temperature (24°C) and a short-day (9 h) photoperiod to prevent flowering. The plants were watered twice daily, once with tap water and once with one-half-strength Hoagland solution. After 4 weeks of growth under these conditions, the plants were gently washed in water to remove the sand adhering to the roots and transferred to a nutrient film technique setup. The plants were placed in channels and quarter-strength Hoagland's nutrient solution (Hoagland and Arnon, 1938) amended with 0.05, 0.075, and 0.1 mM Cd (as $CdSO_4$) was circulated along the plant roots. This setup was maintained under the same greenhouse conditions. Plants were harvested after 14 d and thoroughly washed under running deionized water to remove any trace elements adhering to the tissue. Total fresh weights of the plants were measured before and after the experiment to determine the effect of different concentrations of Cd on growth. Shoot and root tissues were separated and dried at 70°C for 3 d. The dried tissues were weighed and then ground in a Wiley mill.

Glutathione and NPT Analysis

Total NPTs and total glutathione were measured spectrophotometrically in seedlings obtained from seedling ex-

periment A. For total NPT analysis, extracts were prepared according to the method described by Galli et al. (1996), by adding 300 μL of a solution containing 1 M NaOH and 1 mg L^{-1} $NaBH_4$ to 100 mg of homogenized plant sample. The homogenate was centrifuged for 3 min at 13,000*g* at 4°C. Supernatant (300 μL) was acidified by adding 50 μL of 37% HCl. NPT contents were measured spectrophotometrically by adding 20 μL of this solution to 1 mL of of 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, Ellman, 1959), followed by the measurement of the A_{412}. Total glutathione was measured according to the method of Hermsen et al. (1997).

Cys, γ-EC, GSH, and PC levels in the seedlings obtained from experiment B were analyzed by HPLC according to the method of Weber et al. (1999). NPTs were extracted from 100 mg fresh weight of both shoot and root samples using 200 μL of 10% sulfosalicylic acid containing 12.6 mM diethylenetriaminepentaacetic acid. The homogenate was centrifuged, filtered (0.22-μm pore size), and diluted before injection. As a comparison, the homogenate was also used for total NPT measurement spectrophotometrically, as described above. The HPLC equipment included a pump (LC-10AD, Shimadzu, Kyoto), a C_{18} column (250 × 4.6 mm, 5-μm particle size, Altima, Alltech, Deerfield, IL), a precolumn (All-Guard, Alltech), and a 50-μL injection loop. The detection system included an amperometric electrochemical detector with a gold/mercury electrode (model LC-4B, Bioanalytical Systems, West Lafayette, IN). The mobile phase was 9.4 g of monochloroacetic acid and 40 mL of methanol in 1 L of water, pH adjusted to 3.1 to 3.2 with sodium hydroxide pellets. The system was run at a flow rate of 1 mL min^{-1} at an applied voltage of 0.154 V. The electrode was polished and plated with mercury the day before analysis according to the instructions provided. The mobile phase was continuously de-gassed throughout the operation to eliminate interference from oxygen. The detection limit for GSH is 5 pmol.

Measurement of γ-ECS Activity

Protein was extracted from shoots of unstressed seedlings in experiment B. After grinding in liquid nitrogen, extractions were made by adding (2 mL g^{-1} fresh weight) buffer (50 mM potassium phosphate and 1 mM phenylmethylsulfonyl fluoride, pH 8.0), followed by centrifugation at 13,000*g* for 5 min at 4°C. Protein content in the supernatant was determined using the standard Bio-Rad assay kit. For γ-ECS activity, γ-EC formation in a reaction mix was determined with HPLC as described above. The reaction was started by adding 10 μL of the protein extract to 50 μL of γ-ECS assay solution containing 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8.0), 50 mM $MgCl_2$, 20 mM glutamate, 1 mM Cys, 5 mM ATP, 10 mM phosphocreatine, 10 units/mL phosphocreatine kinase, and 0.5 mM dithiothreitol. After 20 and 80 min of incubation at 30°C, 10 μL of the reaction mixture was added to 40 μL of the mobile phase, followed by either immediate HPLC analysis or immediate storage in dry ice for HPLC analysis the same day. γ-EC formation in 60 min was calculated by subtracting the 20-min γ-EC peak from the 80-min γ-EC peak.

Elemental Analysis

Elemental analysis was carried out after acid digestion of dried and ground tissue samples as described by Zarcinas et al. (1987). The concentrations of trace elements in the acid digest were measured by inductively coupled plasma atomic emission spectroscopy (Fassel, 1978). Standards (National Institute of Standard and Technology) and blanks were run with all samples for quality control. Plants that had not been supplied with trace elements were also analyzed for trace element concentrations as a negative control.

Statistical analyses were performed using the JMPIN statistical package (SAS Institute, Cary, NC).

RESULTS

Production and Characterization of Transgenic γ-ECS Plants

Five kanamycin-resistant Indian mustard lines were obtained after transformation with the *gshI* construct, and were designated ECS1, ECS2, ECS3, ECS4, and ECS6. All five plant lines showed a product when PCR was performed using primers directed against the 35S promoter and the *gshI* gene (not shown). Progeny of lines ECS2 and ECS4 showed a kanamycin resistance ratio of 3:1 (100 mg L^{-1} in one-half-strength Murashige and Skoog medium) after self-fertilization of the first generation, indicating a single insert of the *gshI* gene. Homozygous T2 lines from individual T1 plants of lines ECS2 and ECS4 were used for subsequent experiments. These transgenic T1 and T2 plants did not show any distinguishable difference in visual appearance under unstressed conditions compared with the WT plants.

When *gshI* DNA was used as a probe for a northern blot containing total RNA isolated from T2 seedlings of ECS2 and ECS4, both transgenic lines showed a band of the *E. coli gshI* transcript (Fig. 1A). Antiserum raised against *E. coli* γ-ECS was used to analyze transgenic expression in the transgenic lines at the protein level. On western blots, shoot tissues from ECS2 and ECS4 lines were both shown to contain a protein with the same molecular mass as the *E. coli* γ-ECS (64 kD), which reacted with the antiserum (Fig. 1B); no band was detected in WT extract. The expression levels of the *E. coli* γ-ECS protein were similar in ECS4 and in ECS2. Shoots of both ECS2 and ECS4 plants showed approximately 5-fold-increased γ-ECS activity compared with WT shoots (Table I).

γ-ECS Plants Show Improved Cd Accumulation and Tolerance

Two experiments were conducted to test Cd tolerance, one using seedlings and one mature plants. For Cd tolerance, we used root length, which is considered to be a reliable parameter for heavy metal tolerance (Murphy and

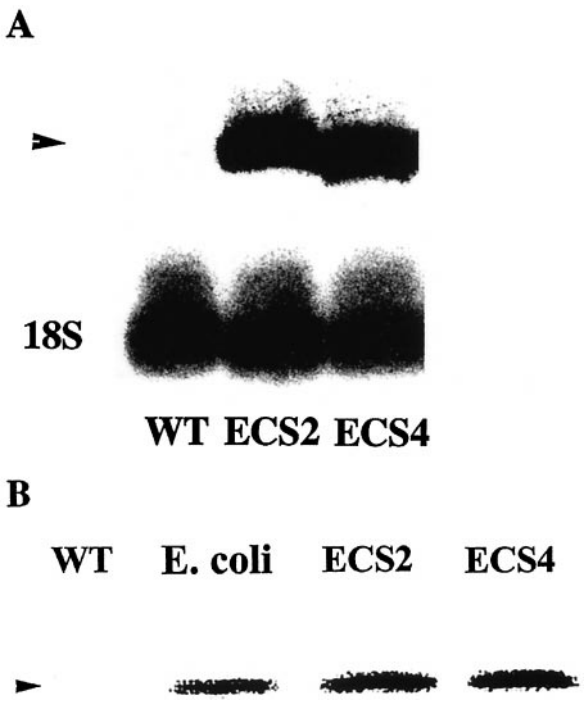

Figure 1. A, Northern blot of total shoot RNA from Indian mustard seedlings. Total RNA samples from WT and transformed (ECS2 and ECS4) Indian mustard were loaded into each lane (10 μg per lane). The probe used to detect γ-ECS mRNA corresponded to an internal 1.85-kb fragment of the *gshI* coding sequence. B, Western blot of seedling extracts from WT and γ-ECS-overexpressing (ECS2 and ECS4) transgenic Indian mustard plants (digitized image). Equal amounts (10 μg) of total protein were loaded in each lane, separated on a 10% SDS-PAGE gel, blotted, and treated with antiserum raised against purified *E. coli* γ-ECS protein. Samples were pooled from 25 seedlings grown on one-half-strength Murashige and Skoog medium. *E. coli* extract was included as a positive control.

Taiz, 1995). After 7 d on agar medium containing 0.15, 0.20, or 0.25 mM $CdSO_4$, the γ-ECS seedlings had longer roots than WT seedlings (Fig. 2A). For example, at 0.20 mM Cd, the roots of ECS4 seedlings were more than 2-fold longer ($P < 0.001$) than those of WT seedlings. Under control conditions, there were no significant differences in root length between the γ-ECS seedlings and the WT seedlings (Fig. 2A). The shoots of the transgenic seedlings were also taller than shoots of WT seedlings after Cd treatment (Fig. 2B).

Table I. *Activities of γ-ECS in shoots from WT and γ-ECS-overexpressing (ECS2 and ECS4, respectively) transgenic Indian mustard plants*

The data represent means ± SD of three separate extractions. *, Significantly different ($P < 0.05$) from WT of the same treatment.

Plant Line	γ-ECS Activity
	nmol mg^{-1} protein min^{-1}
WT	0.137 ± 0.010
ECS2	0.651 ± 0.061*
ECS4	0.673 ± 0.144*

The Cd tolerance experiments with mature plants were performed with plants from transgenic lines ECS2, ECS4, and WT. After 14 d of growth on nutrient solutions containing 0, 0.05, 0.075, or 0.10 mM $CdSO_4$, the γ-ECS plants showed superior Cd tolerance compared with WT: their growth (whole plant biomass) was less inhibited by Cd than the growth of WT plants (Fig. 3), with the exception of

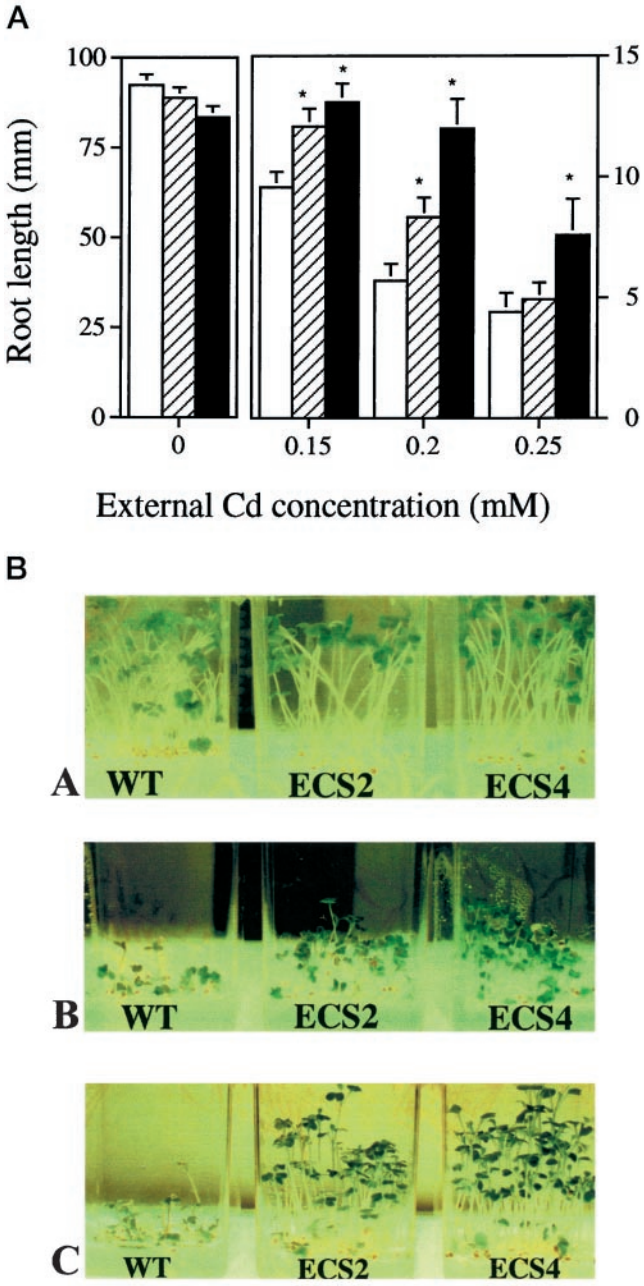

Figure 2. Effect of Cd on the growth of WT (white bars) and GS-overexpressing (ECS2 [hatched bars] and ECS4 [black bars]) Indian mustard seedlings grown for 7 d on agar medium containing 0, 0.15, 0.20, and 0.25 mM $CdSO_4$. A, Root length (the averages ± SE of 40 seedlings); B, seedlings growing in agar medium without Cd for 6 d (A), with 0.2 mM Cd for 6 d (B), and with 0.2 mM Cd for 12 d (C). *, Significant difference ($P < 0.05$) from the WT of the same treatment.

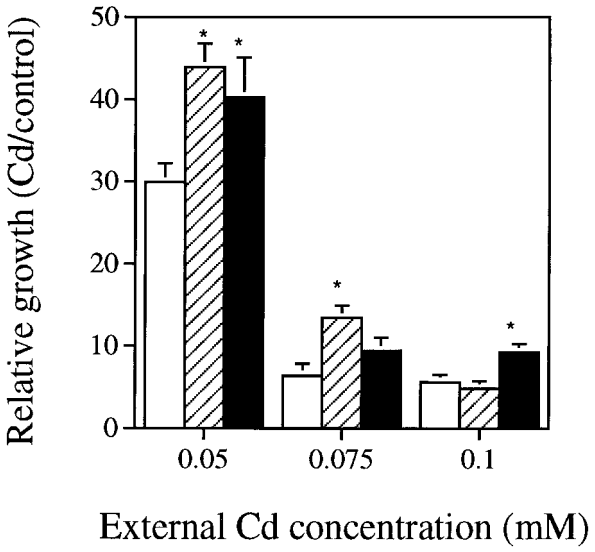

Figure 3. Growth of mature WT (white bars) and γ-ECS-overexpressing (ECS2 [hatched bars] and ECS4 [black bars]) Indian mustard plants treated with 0.05, 0.075, and 0.10 mM $CdSO_4$. Values shown are the average ± SE of 11 replicate plants. Relative growth was calculated as the harvest fresh weight (shoot plus root) minus the fresh weight before treatment, divided by the latter. *, Significant difference ($P < 0.05$) from the WT of the same treatment.

ECS2 at 0.1 mM. At 0.05 mM Cd, the relative growth of ECS2 plants was 44% of that of untreated ECS2 controls, while the relative growth of WT plants was 30%.

The Cd concentrations in plant roots and shoots were determined for the plants obtained from the mature plant experiments. Both ECS2 and ECS4 plants showed higher Cd concentrations in their shoots than WT plants. For example, when grown at 0.05 mM external Cd, the shoot Cd concentrations in ECS4 plants were 90% higher than in WT plants ($P < 0.01$, Fig. 4A). The Cd concentrations in roots of ECS2 and ECS4 plants were also slightly higher than in WT plants, but these differences were not significant (Fig. 4B).

γ-ECS Plants Contain Higher Levels of GSH, PCs, and Total NPTs

To investigate the effect of γ-ECS overexpression on the production of heavy metal binding compounds, total NPTs and glutathione levels were measured spectrophotometrically in shoot samples collected from ECS2, ECS4, and WT seedlings used in seedling experiment A, which were treated with 0.15, 0.20, or 0.25 mM Cd. The glutathione levels were 1.5- to 2.5-fold higher in both γ-ECS lines compared with the WT (Fig. 5A). This difference was true for both Cd-treated and untreated seedlings. ECS2 and

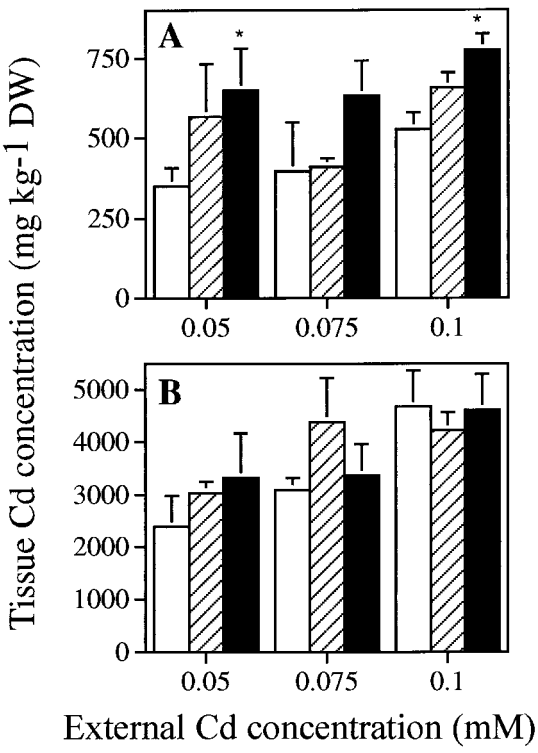

Figure 4. Cd concentration in shoots (A) and roots (B) of WT (white bars) and γ-ECS-overexpressing (ECS2 [hatched bars] and ECS4 [black bars]) Indian mustard plants treated with 0.05, 0.075, and 0.10 mM $CdSO_4$. Values shown are the average ± SE of eight replicate plants. *, Significant difference ($P < 0.05$) from the WT of the same treatment. DW, Dry weight.

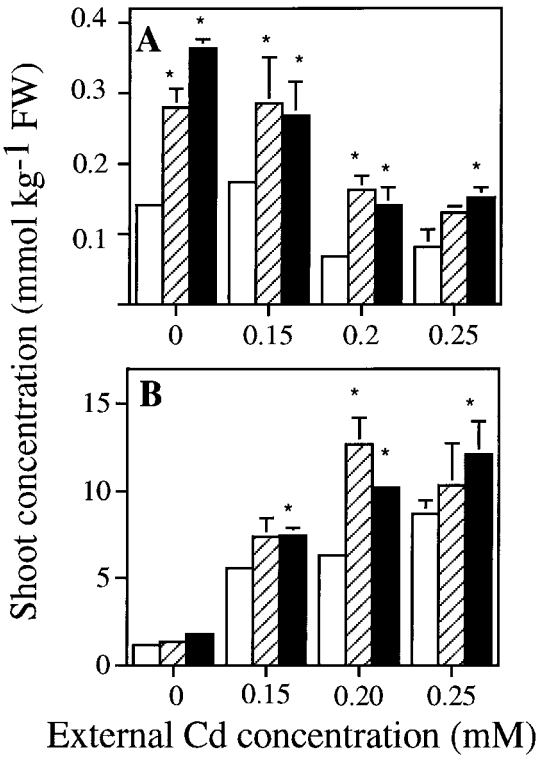

Figure 5. Shoot tissue concentrations of glutathione (A) and total NPTs (B) in WT (white bars) and γ-ECS-overexpressing (ECS2 [hatched bars] and ECS4 [black bars]) Indian mustard seedlings grown in the absence (0) or presence (0.15, 0.20, and 0.25 mM) of $CdSO_4$. Values shown are the average ± SE of three replicate samples from four plants each. *, Significant difference ($P < 0.05$) from the WT of the same treatment. FW, Fresh weight.

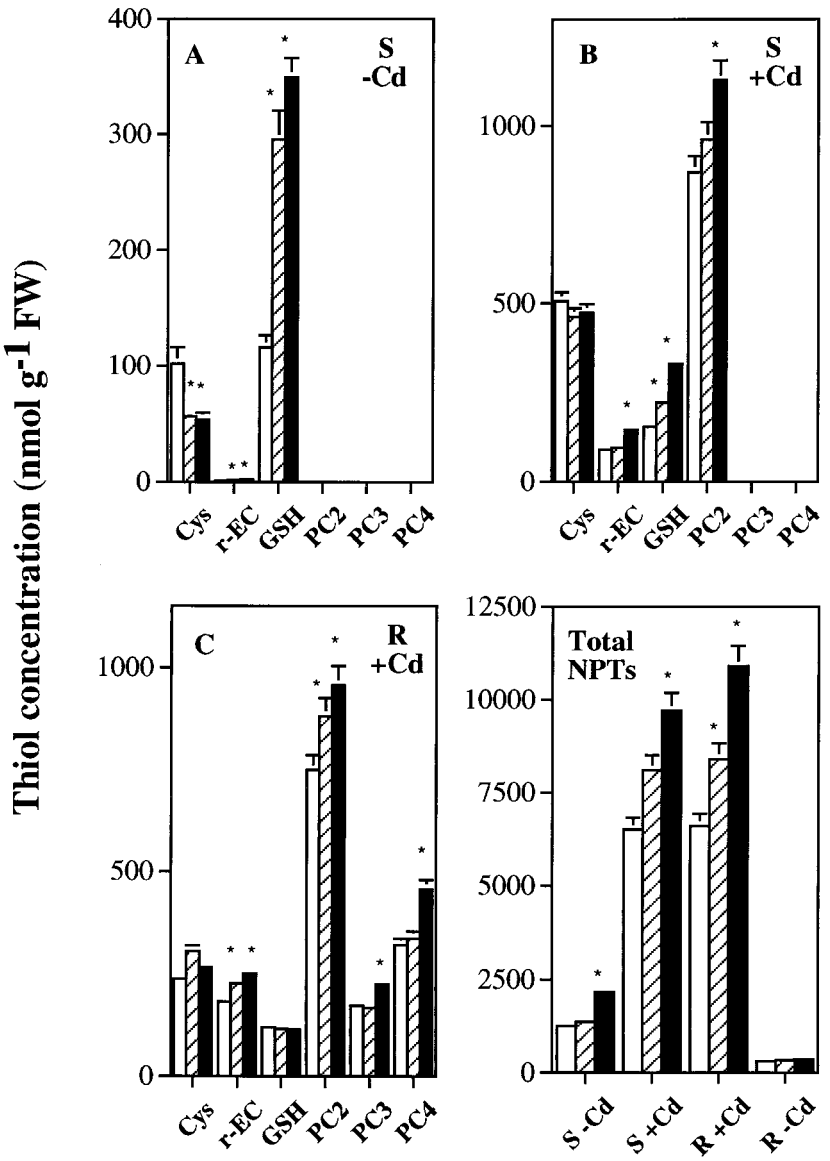

Figure 6. Thiol concentrations in shoots of unstressed seedlings (S, −Cd) (A), in shoots of 0.2 mM Cd treated seedlings (S, +Cd) (B), in roots of 0.2 mM Cd-treated seedlings (R, +Cd) (C), and total NPTs in shoots or roots of 0.2 mM Cd-treated or unstressed seedlings from experiment B. White bars, Control; hatched bars, ECS2; black bars, ECS4. *, Significant difference ($P < 0.05$) from the WT of the same treatment. FW, Fresh weight.

ECS4 plants had slightly higher levels of total NPTs than WT under control conditions (1.36, 1.80, and 1.16 μM g^{-1} fresh weight, respectively), but this difference was not significant. In contrast, the total NPT levels in Cd-treated γ-ECS seedlings were significantly higher (approximately 1.5-fold) than in WT seedlings (Fig. 5B).

The higher total NPT concentrations in both roots and shoots were also observed in 0.2 mM Cd-treated γ-ECS seedlings from experiment B (Fig. 6). In this experiment, individual NPTs including GSH, Cys, γ-EC, and PCs were determined with HPLC. As described above, shoot GSH concentrations were higher in ECS2 and ECS4 seedlings

Table II. *Concentrations (mg kg^{-1} dry weight) of total Ca, Fe, Mn, P, and S in shoots of mature ECS2, ECS4, and WT Indian mustard plants grown in control (unstressed, −Cd) and 0.1 mM Cd (+Cd) in the mature plant experiment*

The data represent means ± SD of six replicate plants. ND, Not determined. *, Significantly different ($P < 0.05$) from WT of the same treatment.

Element	−Cd			+Cd		
	WT	ECS2	ECS4	WT	ECS2	ECS4
Ca	32,785 ± 5,947	ND	27,759 ± 5,506	20,247 ± 2,801	27,787 ± 4,710	36,504 ± 3,934*
Fe	72.5 ± 36.4	ND	102.3 ± 33.7	30.6 ± 3.25	78.3 ± 43.6*	50.9 ± 12.2
Mn	32.1 ± 22.8	ND	22.9 ± 4.30	11.7 ± 0.98	13.6 ± 1.89	18.4 ± 1.89*
P	4,893 ± 540	ND	4,778 ± 500	3,041 ± 159	3,256 ± 186	3,811 ± 488
S	9,385 ± 1,200	ND	7,598 ± 1,112	6,077 ± 718	7,610 ± 577*	10,803 ± 1,956*

than in WT seedlings (under both 0 and 0.2 mM Cd treatment, Fig. 6, A and B). However, root GSH concentrations were similar among all three lines (Fig. 6C) under Cd treatment.

A small, but significantly greater amount of γ-EC was detected in untreated ECS2 (1.6 nmol g^{-1} fresh weight) and ECS4 (2.1 nmol g^{-1}) seedlings compared with WT seedlings (0.8 nmol g^{-1}) (Fig. 6A). γ-EC concentration was also significantly higher in roots of Cd-treated ECS2 (228 nmol g^{-1}) and ECS4 (252 nmol g^{-1}) seedlings and in shoots of Cd-treated ECS4 seedlings (145.7 nmol g^{-1}) compared with WT (root 183 nmol g^{-1} and shoot 89.9 nmol g^{-1}). ECS2 shoots (95.8 nmol g^{-1}) showed an intermediate γ-EC concentration. While Cys concentrations were significantly lower in shoots of ECS2 and ECS4 seedlings than in shoots of WT seedlings under unstressed conditions, they were similar in shoots of ECS2, ECS4, and WT seedlings under Cd stress (Fig. 6).

Both Cd-treated γ-ECS and WT seedlings accumulated PC2 in shoots, and PC2, PC3, and PC4 in roots. ECS4 shoots (1128 nmol μM g^{-1}) showed a significantly ($P = 0.014$) higher PC2 level than WT shoots (870.8 nmol μM g^{-1}) (Fig. 6B). PC2 concentration in ECS2 (961.5 nmol μM g^{-1}) was intermediate (ECS2/WT, $P = 0.100$). ECS4 roots also had significantly higher concentrations of PC2, PC3, and PC4 (Fig. 6C). ECS2 roots only showed higher levels of PC2 compared with WT roots.

Effects of γ-ECS Overexpression on Mineral Nutrient Levels

To investigate the effect of Cd on tissue mineral nutrient levels, the concentrations of B, Ca, Cu, Fe, K, Mg, Mn, Mo, P, S, and Zn were determined in shoots and roots of mature γ-ECS and WT plants treated with 0 or 0.1 mM Cd. Cd treatment had significant effects on the nutrient levels of some elements in the plants (Table II). In WT plants, the levels of Ca, Fe, Mn, P, and S were significantly lower under 0.1 mM Cd treatment than under non-Cd conditions (levels of B, Cu, Mg, K, Mo, and Zn were not affected by Cd). Interestingly, the levels of Ca, Fe, Mn, P, and S were all significantly higher in Cd-treated ECS4 plants than in Cd-treated WT plants (Table II). ECS2 also had higher concentrations of Ca, Fe, Mn, P, and S than WT but only Fe and S were statistically significant (Table II). In the absence of Cd there were no significant differences between γ-ECS and WT plants with respect to tissue concentrations of any of these elements except for Cu and Zn that were higher in the γ-ECS plants (data not shown). The root concentrations of all elements tested did not show any significant differences between γ-ECS and WT plants.

DISCUSSION

Overexpression of the *E. coli gshI* gene in Indian mustard increased γ-ECS activity compared with WT. The activity of this enzyme in transgenic seedling shoots was 5-fold greater than in WT shoots (Table I). The *E. coli* protein was most probably targeted to the chloroplast, as the gene construct contained the pea rbcS sequence, coding for the chloroplast transit peptide of the rbcS. Using the same construct, Noctor et al. (1998a) concluded that the *E. coli* protein was present in the chloroplast of transgenic poplar. In plants treated with Cd, overexpression of the *E. coli gshI* gene increased the production of γ-EC, the direct product of the γ-ECS enzyme, as well as GSH and PCs farther down the pathway. In unstressed plants (i.e. those not treated with Cd), there was increased production of γ-EC and GSH but not PCs, because PC synthase requires Cd for activation (Zenk, 1996). Both γ-EC and GSH concentrations were 2 to 3 times higher in the transgenic lines, ECS2 and ECS4, than in the WT (Fig. 6); this compares to 10- to 15-fold increases of γ-EC levels in poplar (Arisi et al., 1997; Noctor et al., 1998a).

Our results show that plants overexpressing γ-ECS exhibited increased Cd tolerance and accumulation. It is presumed that the increased Cd tolerance and accumulation were due to the enhanced PC production by γ-ECS plants, which should lead to a greater capacity to detoxify and sequester Cd (Steffens, 1990; Zenk, 1996). However, it is possible that the increased levels of GSH in γ-ECS plants may have also contributed to the increased tolerance in γ-ECS plants. GSH is a major component of the active oxygen scavenging system of the cell (Noctor and Foyer, 1998), and the relatively high levels of GSH in the tissues of the γ-ECS plants could have conferred increased Cd tolerance by protecting cells from metal-related oxidative stress damage (Gallego et al., 1996; Weckx and Clijsters 1996, 1997). On the other hand, higher GSH levels did not confer better oxidative stress resistance in transgenic poplar (Noctor et al., 1998b), and higher GSH levels may even make transgenic plants more sensitive to oxidative stress, as recently observed in γ-ECS overexpressing tobacco (Creissen et al., 1999). Therefore, it is uncertain whether GSH can protect oxidative damage caused by heavy metals. It is also possible that GSH detoxifies Cd by directly forming a GSH-Cd complex; a $Cd(GS)_2$ complex was found in Cd-treated yeast (Li et al., 1997).

Unlike shoots, roots of γ-ECS and WT plants had similar Cd concentrations even though PC levels were higher in roots of γ-ECS plants than in roots of WT plants. One explanation for this is that roots have much higher Cd concentrations than shoots (at least 6 to 10 times higher), with most of the Cd being bound to the root cell wall (Salt et al., 1997). Thus, any increase in Cd sequestration due to greater PC complexation in the γ-ECS plants compared with WT may have been masked by the very high Cd concentrations in roots.

Earlier studies have shown that γ-ECS is rate limiting for GSH synthesis in "normal" plants (Noctor et al., 1997, 1998b), i.e. those not stressed with Cd. Our results support this view because overexpression of γ-ECS in unstressed Indian mustard plants led to increased GSH levels. Under conditions of Cd stress, PC synthase is activated by Cd (Goldsbrough, 1998). Since overexpression of γ-ECS increased γ-EC, GSH, and PC levels in transgenic seedlings, it would appear that γ-ECS is rate limiting for both GSH and PC production in Cd-stressed plants.

In an earlier paper we concluded that GS was not rate-limiting for GSH production in unstressed Indian mustard

plants, since overexpression of the GS enzyme did not increase GSH levels (Zhu et al., 1999). This is consistent with the results presented here, which suggest that γ-ECS is rate-limiting for GSH production. Under conditions of Cd stress, however, the earlier research showed that overexpression of GS led to increased GSH and PC levels, suggesting that GS was limiting GSH production under those conditions. We explained this by suggesting that in Cd-treated plants, Cd activated γ-ECS so that γ-EC accumulated and GS became rate limiting (Zhu et al., 1999). This view was supported in the present work because Cd treatment increased γ-EC concentration 50- to 100-fold in shoots of both WT and γ-ECS seedlings (Fig. 6, A and B).

Cd-caused accumulation of γ-EC has also been observed in maize seedlings (Rauser et al., 1991). The fact that γ-EC levels were so high, even in the γ-ECS seedlings, suggests that GSH production in the Cd-treated plants was limited by GS. On the other hand, the observations that GSH and PC levels were increased by overexpression of γ-ECS and the Cd-caused increase in Cys levels (both in WT and ECS plants, Fig. 6, A and B) suggest that γ-ECS is rate-limiting as well. Thus, it appears that the two enzymes co-limit GSH production under Cd stress. The observation that Cys synthesis was stimulated by Cd treatment and by overexpression of γ-ECS (see following paragraph) suggests that Cys synthesis does not rate-limit GSH production under these conditions.

The elevated levels of total shoot S and total NPTs in the γ-ECS plants suggests that overexpression of γ-ECS results in enhanced S assimilation. In poplar plants overexpressing the same γ-ECS construct, up-regulation of Cys synthesis was observed: the γ-ECS-overexpressing poplar plants showed 2- to 4-fold increased in vitro activities of APS reductase and Ser acetyltransferase (Noctor et al., 1998b). Cys concentrations were also increased 5- to 8-fold by Cd treatment in both γ-ECS and WT shoots. In addition to γ-ECS, Cd may induce several genes encoding enzymes of the S assimilation pathway, such as ATP sulfurylase and APS reductase (Heiss et al., 1999); the transcript levels of these two enzymes were also found to be enhanced by Cd in Indian mustard (Lee and Leustek, 1999). Thus, the S assimilation pathway may be enhanced by both overexpression of γ-ECS and by Cd treatment. Both effects may be mediated by an increase in γ-EC synthesis.

In conclusion, this study shows that the γ-EC enzyme is rate-limiting for the synthesis of GSH in Cd-stressed and unstressed plants, and that overexpression of γ-ECS increased the production of γ-EC, GSH, total NPTs, and PCs in Cd-treated plants. We conclude that the greater Cd tolerance and accumulation in the transgenic plants compared with WT were due primarily to the increased level of PCs. Thus, overexpression of γ-ECS appears to be a promising strategy for enhancing the efficiency of Cd phytoextraction from polluted soils and wastewater.

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