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Alteration of Thiol Pools in Roots and Shoots of Maize Seedlings Exposed to Cadmium[1]

Adaptation and Developmental Cost

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ABSTRACT

Roots of intact 5-day-old maize (*Zea mays* L.) seedlings were exposed to 3 micromolar Cd during a 7-day period. Cysteine, γ-glutamylcysteine, glutathione (GSH), and Cd-induced acid-soluble thiols (ASTs), including phytochelatins, were quantified in roots and shoots. Adaptation to Cd and its cost to seedling development were evaluated by measuring Cd content, tissue fresh weight, and rate of root elongation. Roots contained 60 to 67% of the Cd in the seedlings between 4 and 7 days of exposure. Exposure to Cd decreased the fresh weight gain in roots from day 4 onward without affecting the shoots. Between days 1.5 and 3.5 of Cd treatment, roots elongated more slowly than controls; however, their growth rate recovered thereafter and exceeded that of controls. Exposure to Cd did not appreciably affect the concentration of cysteine in the seedlings. However, the initial low concentration of γ-glutamylcysteine increased (after a lag of 6 hours in roots and 2 days in shoots), reaching a plateau by day 6 at 28.5 nanomoles per gram of fresh weight in roots and by day 5 at 19.1 nanomoles per gram of fresh weight in shoots. During the first 9 hours of Cd exposure, the concentration of GSH in roots decreased dramatically (at 31.6 nanomoles per gram of fresh weight per hour) and thereafter decreased more slowly than in controls. The depletion of GSH in the roots (366 nanomoles per gram of fresh weight) matched the synthesis of ASTs (349 nanomoles per gram of fresh weight) during the first 48 hours. The concentration of ASTs in roots increased steadily thereafter to reach 662.2 nanomoles per gram of fresh weight by 6 days of Cd exposure. In shoots, Cd had little influence on the concentration of GSH, but ASTs still accumulated to 173.3 nanomoles per gram fresh weight after 5 days. The molar ratio of thiols in ASTs to Cd increased to a maximum of 10.24 in roots after 4 hours and of 4.25 in shoots after 2 days of Cd exposure. After 4 days, the ratio reached a plateau of approximately 2 in roots and between 2 and 3 in shoots, as if a steady state of Cd chelation had been achieved in both organs. The plateau coincided with recovered root elongation or an adaptation to Cd. The reduced fresh weight gain of the roots during this time, however, indicated that the synthesis of Cd-induced thiols was at a cost to root development.

Plants respond to Cd stress by synthesizing metal-binding polypeptides analogous to the metal-binding metallothioneins of animals and certain fungi (15, 23). These cysteine-rich polypeptides are class III metallothioneins with the structure of PCs[3] in which the γEC portion is repeated two to 11 times (4, 8, 10, 25). We refer to such polypeptides here as PCs, knowing the reservations of this nomenclature (15, 23). The enzyme PC synthase adds the γEC portion of GSH to GSH itself and to elongating PCs (2). Using a crude extract from fission yeast, Hayashi *et al.* (6) corroborated the PC synthase activity and showed that γEC, an intermediate in the pathway of GSH biosynthesis, was itself polymerized into desGly polypeptides and PCs.

Adaptation of plants to otherwise cytotoxic amounts of Cd is related to their ability to produce PCs and then to form Cd-binding complexes or aggregates from PCs of different chain lengths (5, 8). Cd is chelated through the thiol group of Cys in the PCs with the molar ratio of thiol to Cd being 2 to 3 (4, 15, 17). The presence of acid-labile sulfide increases the stability of Cd-binding complexes in fission yeast (11, 18) and may form the basis of Cd tolerance in *Silene vulgaris* (28). High amounts of reduced sulfur are thus needed by plants to provide sulfide for synthesis of Cys required in formation of PCs and Cd-binding complexes. Indeed, the rate of assimilatory sulfate reduction increases in roots of Cd-exposed maize seedlings (12). Reduced sulfur via Cys is also used for general protein synthesis in the developing plant.

As plants respond to Cd exposure through synthesis of PCs, there is an increased demand for GSH. Exposure of seedlings and cell cultures to Cd results in a rapid and extensive decline in the pool of GSH (1, 5, 13, 21, 27). Such Cd-induced declines in GSH match the early PC biosynthesis in cell cultures of *Rauwolfia serpentina* (5) and *Datura innoxia* (1) and in maize roots (27). Supply of GSH can be controlled in various ways. One is by local biosynthesis through the sequential action of γEC synthetase (EC 6.3.2.2) joining Glu to Cys to form γEC, to which Gly is then added by GSH synthetase (EC 6.3.2.3) (7, 19). Cd inhibits γEC synthetase (7), and Cd-resistant tomato cell lines have greater activity of γEC synthetase than do sensitive cells (26). The effect of Cd

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[3] Abbreviations: PCs, phytochelatins or poly(γ-glutamylcysteinyl)glycines; γEC, γ-glutamylcysteine; ASTs, acid-soluble thiols other than Cys, γEC, and GSH.

on GSH synthetase is controversial; more enzyme activity is extractable from Cd-treated pea plants (20), yet the enzyme from *Petrosilenum crispum* is inhibited by Cd and the inhibition can be relieved by dialysis (L. Bergmann, personal communication). Supply of GSH to roots of maize seedlings through influx via phloem translocation is not inhibited by Cd (16). The concentration of Cys may also control supply of GSH as suggested for *Datura innoxia* cells (1). The catabolism of GSH in plant cells differs from that in animal cells, proceeding through the formation of γEC, 5-oxo-proline, and finally Glu with the successive release of Gly and Cys (19).

Most of our knowledge on Cd-binding complexes and pools of low mol wt thiols in plants comes from cell cultures and short-term exposures of roots of intact plants (15, 23). Several ASTs, including PCs, appeared in maize roots exposed to Cd up to 48 h (27). In a study of the distribution and translocation of radioactive thiols in maize seedlings, it was noticed that endogenous γEC accumulated in roots and shoots during a 24-h exposure to Cd (16). The experiments reported here were designed to measure the concentrations of Cys, γEC, and GSH in roots and shoots of intact maize seedlings during an extended 7-d period of exposure to 3 μM $CdSO_4$. The appearance of Cd-induced ASTs was also quantified in both organs. The process of Cd adaptation during the development of the root was evaluated by comparing the levels of these thiols to the rate of root elongation, the tissue fresh weight, and the content of Cd.

MATERIALS AND METHODS

Plant Material

Caryopses of maize (*Zea mays* L., hybrid 37701) were obtained from Cargill Hybrid Seeds Ltd. (Princeton, Ontario, Canada) and germinated in moist paper towels as previously described (27). After 3 d, seedlings were transplanted into aerated nutrient solution (50 seedlings in 4 L of one-half strength Hoagland solution) and grown at a controlled temperature (22–23°C) with a 16-h/d light period beginning at 07:00 h (27). The primary roots were exposed to 3 μM of $CdSO_4$ in nutrient solution from 5 to 12 d after planting. Plant material was collected after 2, 4, 6, 9, and 12 h and then daily up to 7 d at 10:00 h, the starting time of exposure. Nutrient solutions of both control and treated plants were replaced daily. Root lengths were measured after 12 h and just before the daily collections.

At the time of collection, the roots of seedlings were immersed for 10 min in ice-cold 5 mM $CaCl_2$ solution to displace extracellular Cd (14). The roots were then blotted to remove excess solution. The primary and adventitious roots were cut from the shoots, the tissues were packed into small aluminum pouches, the fresh weights were measured, and the tissues were frozen in liquid N_2. Samples were stored at −80°C for up to only 6 weeks (significant degradation of individual thiol compounds was found after 3 months of storage). For the early collections, the separate tissues from 10 seedlings were pooled into one sample; the roots of four seedlings and the shoots of two were sufficient by day 7. Seedlings were grown on three occasions during a 2-month period; on one occasion two replicates were harvested, giving a total of four replicates.

Extraction Procedure

The procedure for extracting thiols was similar to the one described by Rauser *et al.* (16) with minor modifications. Frozen tissues were homogenized in ice-cold 0.1 N HCl-1 mM EDTA in small mortars and pestles kept on ice. For primary roots and for shoots, the extraction volume was equal to twice the fresh weight and five times the fresh weight for adventitious roots. Homogenates were centrifuged at 4°C and 6000 rpm for 5 min and the supernatants recentrifuged for 10 min. The total volume of these crude extracts was corrected afterward for the contribution of tissue fluids by adding 0.9 times the fresh weight.

For quantification of Cys, γEC, and GSH, an 80-μL aliquot of crude extract was reduced for 20 min at room temperature with 130 μL of 100 mM 2(*N*-cyclohexylamino)-ethanesulfonic acid (pH 9.3) and 5 μL of 40 mM DTT. The thiols were then derivatized in dim light (15 min at room temperature) by adding 20 μL of 30 mM monobromobimane (Calbiochem, La Jolla, CA) in acetonitrile. The reaction was stopped by adding 165 μL of 0.25% (v/v) methanesulfonic acid followed by centrifugation at 6000 rpm for 15 min. Derivatized thiol solution (50 μL) was injected onto an RP-HPLC column. Recoveries ranged between 90 and 100% as obtained previously (16). Three standard mixtures of Cys, γEC (obtained from Nakarai Chemicals Ltd., Kyoto, Japan), and GSH at various concentrations encompassing those found in extracts were treated as above and used to plot the regression lines at each analysis.

Analytical Procedures

The bimane derivatives of Cys, γEC, and GSH were separated on an RP-18 column (250 × 4.6 mm i.d., 5-μm particles, Hypersil-ODS; Chromatographic Sciences Company, Montreal, Quebec, Canada) coupled to a precolumn (2 × 20 mm, 30- to 40-μm pellicular Perisorb RP-18) with a 0.5-μm inlet frit. The eluting conditions were adapted from Schupp and Rennenberg (22) and optimized for the HPLC system available (Dionex BioLC, Dionex, Mississauga, Ontario, Canada). The flow rate was 1 mL/min with the column at 40°C. The eluant was 0.25% (v/v) acetic acid in water (pH 3.9) containing 11.5% (v/v) methanol. After isocratic elution for 21 min, the column was washed by increasing the concentration of methanol to 90% (v/v) for 1 min and holding it at this concentration during 5 min before returning to the initial conditions. After 8 min of equilibration, the next sample was injected automatically (Autosampler SP 8875; Spectra-Physics, San Jose, CA).

The bimane derivatives were detected fluorimetrically at wavelengths >425 nm after excitation at 360 nm (model 420 fluorescence detector; Waters, Mississauga, Ontario, Canada). The signals were stored and processed by an IBM PS2 computer. Under these conditions the bimane derivatives of Cys, γEC, and GSH eluted at 10.0, 14.4, and 18.6 min, respectively. Interfering peaks did not contribute to peak integration. The signal to baseline ratio was >3 after samples containing amounts as little as 1 ng of either Cys or γEC were injected. Typical equations of regression lines and the coefficients of correlation (R^2) were as follows: Cys (quantity = response ·

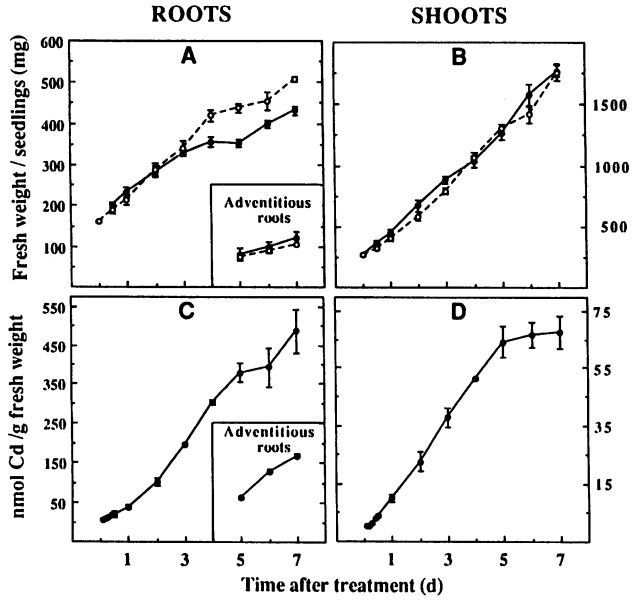

Figure 1. Fresh weight and Cd contents of roots (A and C) and shoots (B and D) of maize seedlings. Roots of 5-d-old seedlings were exposed to Cd concentrations of 0 (○) and 3 μM (●) for 7 d. Note the different scales used for roots and shoots. Points, Means of four replicates; vertical lines exceeding the symbols, SE.

$6.975\ 10^{-5} + 0.7242$; $R^2 = 0.999$); γEC (quantity = response · $7.225\ 10^{-5} + 0.8691$; $R^2 = 0.999$); GSH (quantity = response · $6.579\ 10^{-5} - 43.45$; $R^2 = 0.999$).

ASTs in crude extracts were also quantified by HPLC analysis of 250-μL samples with a postcolumn derivatization procedure (5,5′-dithiobis(2-nitrobenzoic acid) [Ellman's reagent]) as previously described (27). ASTs are defined operationally as those ASTs eluted with 0 to 20% acetonitrile during HPLC analysis; Cys, γEC, and GSH are excluded. Concentrations of Cd in crude extracts were measured by atomic absorption spectroscopy (model AA-475; Varian Inc., Georgetown, Ontario, Canada).

Mann-Whitney tests were used to test the differences between mean values. A probability level of ≤0.05 was considered statistically significant.

RESULTS

Significant morphological changes appeared in the shoots and roots of intact control seedlings during the 7-d time course. At the beginning, the 5-d seedlings had shoots with the first leaf fully expanded and the second one unfurling. Seven days later, the second leaf was fully expanded and the third was appearing. At day 5, the roots comprised a single branched primary root system which grew further during the time course. Adventitious roots emerged 7 to 8 d after planting and reached the nutrient solution 1 d later. Exposure to 3 μM Cd did not change the appearance of the seedlings.

The concentrations of Cd in maize seedlings and their fresh weights are presented in Figure 1. The primary root system in controls enlarged uniformly for 4 d into the time course (now 9-d-old seedlings) and then entered a slower phase that coincided with the development of adventitious roots (Fig. 1A). The fresh weight of Cd-exposed roots increased like the controls for 3 d but slowed 1 d earlier and was significantly lower for the last 4 d of the time course. The gain in fresh weight of shoots was not influenced by Cd (Fig. 1B). The uptake of Cd by roots had no apparent lag phase and proceeded at a rate of 3.91 nmol Cd g^{-1} fresh weight h^{-1} from day 2 to day 5 (Fig. 1C). Adventitious roots acquired Cd at an intermediate rate of 2.15 nmol Cd g^{-1} fresh weight h^{-1}. At any one time, the concentration of Cd in shoots was much less than in roots, with an initial lag phase and a slower rate of Cd acquisition until day 5 (0.56 nmol Cd g^{-1} fresh weight h^{-1}), thereafter entering a plateau (Fig. 1D). Whereas shoots contained about 50 μmol of Cd g^{-1} fresh weight after 4 d of exposing seedlings to Cd, roots reached the same concentration between 2 and 3 d. The distribution of Cd between roots and shoots varied between 60 and 73% from 12 h to 7 d of exposure, as the net Cd content increased in the seedlings.

The rate of elongation of control roots was approximately 2.65 mm h^{-1} from 1.5 d before treatment to 0.75 d after and decreased to 1.56 mm h^{-1} after 6.5 d (Fig. 2). Between 12 and 24 h of exposure to Cd, the rate of elongation was slightly enhanced and then reduced more than controls. However, the rate of elongation of Cd-treated roots increased again after 2.5 d and reached a plateau at a higher level than the controls from 4.5 d onward.

Concentrations of Cys decreased dramatically in roots during the 2 d before treatment but much less in shoots (legend Fig. 3). By 1 d in the time course, the concentrations in both roots and shoots were relatively low (Fig. 3, A and B). Cd slightly increased the concentration of Cys in roots after 12 h of Cd exposure and in shoots between 4 and 5 d. No effect of

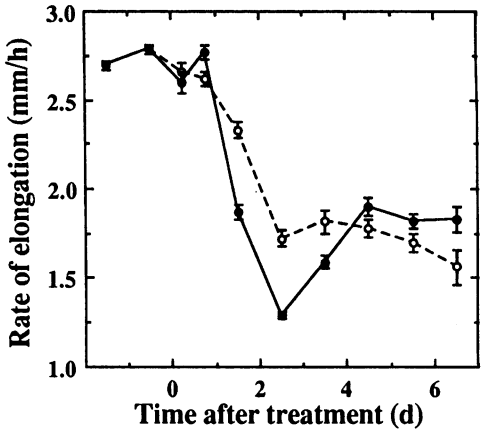

Figure 2. Rate of elongation of primary roots of maize seedlings. Rates are plotted in the middle of the 12- or 24 h-period during which growth occurred. The lengths of the primary root of 200 seedlings were measured 2 and 1 d before half of the plants were exposed to Cd concentrations of 0 (○) and 3 μM (●) for 7 d. Measurements were repeated at 12 and 24 h and daily thereafter for all roots. Because some seedlings (n = 4–10) were harvested after root length measurements, the number of roots making up the means (points) decreased from 90 at day 0 to 40 after 7 d. Vertical lines exceeding the symbols, SE.

Cd on the concentrations of Cys in adventitious roots was measured (Table I).

A net accumulation of γEC to 3.24 nmol g^{-1} fresh weight occurred in roots as soon as 6 h after Cd exposure (Fig. 3C), but an increase in shoots (to 7.4 nmol g^{-1} fresh weight) was measured only after 2 d (Fig. 3D). Subsequently, the concentrations of γEC increased with time in both roots and shoots, reaching a plateau by 6 d. Adventitious roots exposed to Cd also accumulated γEC (Table I).

During the 2 d before Cd exposure, GSH concentrations decreased dramatically in shoots and less so in roots (legend Fig. 3). The concentrations of GSH continued to decrease in both roots and shoots of controls during the time course (Fig. 3, E and F). Cd exposure dramatically enhanced (by 6.9-fold) this pattern in roots during the first 9 h of exposure (Fig. 3E). A linear decrease of GSH at a rate of 31.6 nmol g^{-1} fresh weight h^{-1} was observed in Cd-exposed roots *versus* 4.5 nmol g^{-1} fresh weight h^{-1} in controls. However, between 12 h and 3 d, the rate of GSH decrease in Cd-exposed roots was about 66% of that found in control roots and even smaller from day 4 to day 7. Differences in concentrations of GSH in shoots between controls and Cd-exposed seedlings were less obvious, because only a transitory effect was measured 2 d after exposing seedlings to Cd (Fig. 3F). The lower concentration of GSH in Cd-exposed adventitious roots at day 5 of the time course was also transitory, because considerable recovery occurred by day 7 (Table I).

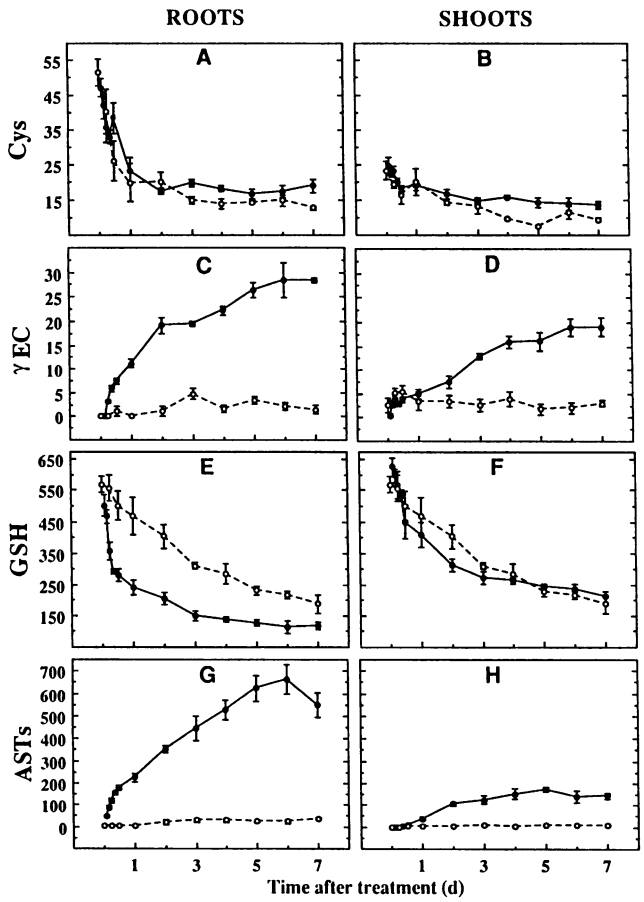

Figure 3. Concentrations of Cys, γEC, GSH, and ASTs in roots (A, C, E, and G) and shoots (B, D, F, and H) of maize seedlings. Roots of 5-d-old seedlings were exposed to Cd concentrations of 0 (○) and 3 μM (●) for 7 d. Thiol concentrations (nmol·g^{-1} fresh weight) in control seedlings 2 and 1 d before Cd exposure were 311.1 ± 5.7 and 163.4 ± 10.5 for Cys in roots, 46.9 ± 8.9 and 35.1 ± 1.3 in shoots; 976.3 ± 26.7 and 856.3 ± 29.9 for GSH in roots, 1743.5 ± 136.6 and 1047.7 ± 14.4 in shoots, respectively. γEC at 1.9 ± 0.3 nmol·g^{-1} fresh weight was detected only in shoots 1 d before Cd exposure. ASTs at 1.68 ± 0.14 and at 2.19 ± 0.21 nmol·g^{-1} fresh weight were detected only in roots 2 and 1 d before Cd exposure. The scales used for each compound are the same for roots and shoots. Points, Means of four replicates; vertical lines exceeding the symbols, SE.

Among Cys, γEC, and GSH of controls, the highest proportion throughout the time course was as GSH, followed by Cys, and then γEC (Fig. 3). By 7 d of Cd exposure, the proportion as γEC increased to 17.3% in roots, to 7.8% in shoots, and to 6.3% in adventitious roots.

ASTs represent the pool of peptides induced by Cd exposure. In roots, this pool includes PCs (15, 23) and at least 10 other thiols (about five in shoots) separated by baseline resolution during HPLC quantification (our unpublished data). Most of these other thiols appear to contain only Cys and Glu. The most abundant thiols have been purified, and comprehensive amino acid analyses are in progress. Under these circumstances, all thiols induced by Cd were combined and designated ASTs. The concentration of ASTs in Cd-exposed roots increased linearly during the first 9 h at a rate of 15.0 nmol g^{-1} fresh weight h^{-1}, after which their biosynthesis slowed down to a rate of 3.7 nmol g^{-1} fresh weight h^{-1} between 12 h and 5 d (Fig. 3G). Shoots of Cd-exposed seedlings also produced some ASTs, as did roots; the accumulation started after a 12-h lag phase, increased linearly to 2 d at 2.7 nmol g^{-1} fresh weight h^{-1} and slowed down toward the end of the treatment (Fig. 3H). Adventitious roots accumulated ASTs at a rate of 5.5 nmol g^{-1} fresh weight h^{-1} between 5 and 7 d (Table I). As described earlier (27), roots of controls contained low concentrations of ASTs mostly as PCs. The amount in primary roots increased from 1.9 nmol g^{-1} fresh weight in 5-d-old seedlings to 34.8 nmol g^{-1} fresh weight 7 d later (Fig. 3G). A similar pattern occurred in control adventitious roots (Table I), and extremely low concentrations of ASTs (2.8 nmol g^{-1} fresh weight at 12 h to 9.4 nmol g^{-1} fresh weight at day 7) were measured in control shoots (Fig. 3H).

At the start of treatment, the 5-d-old seedlings had no detectable γEC and virtually all of total thiols (total thiols = Cys + γEC + GSH + ASTs) were Cys and GSH. After 7 d of Cd exposure, the sum of Cys, γEC, and GSH accounted for merely 23.1% of total thiols in primary roots, for 63.0% in shoots, and for 38.6% in adventitious roots. The concentration of total thiols expressed on a fresh weight basis decreased in control roots by 62.0% and in control shoots by 60.6% during the 7-d time course, yet it increased by 14.5% in Cd-exposed roots and decreased by only 39.3% in their shoots (Fig. 3). However, the amount of total thiols expressed per root initially decreased in Cd-exposed roots (Fig. 4A), mainly due to the depletion of GSH (Fig. 3E), but later exceeded the controls. The amount of total thiols increased in shoots of

Table I. *Concentrations of Cys, γEC, GSH, and ASTs in Adventitious Roots of Control and Cd-Exposed Maize Seedlings*

Adventitious roots emerged from the base of the leaves between 3 and 4 d after starting the treatment and reached the nutrient solution 1 d later. Concentrations are expressed on a fresh weight basis. Mean ± SE of three replicate experiments. nd, Not detectable.

Compound	Time after Starting the Treatment (d)					
	5		6		7	
	Control	Cd-exposed	Control	Cd-exposed	Control	Cd-exposed
	nmol·g⁻¹					
Cys	15.4 ± 0.4	14.8 ± 2.6	19.3 ± 3.2	25.4 ± 1.8[a]	14.4 ± 1.8	18.6 ± 1.4
γEC	nd	8.5 ± 1.8[a]	nd	16.2 ± 2.1[a]	2.4 ± 0.6	18.8 ± 1.7[a]
GSH	471.1 ± 36.3	250.7 ± 15.7[a]	419.4 ± 48.7	321.4 ± 2.8[a]	305.5 ± 37.0	259.2 ± 11.0
ASTs	2.5 ± 2.5	129.8 ± 14.2[a]	2.7 ± 0.6	368.6 ± 12.8[a]	16.4 ± 3.7	412.3 ± 40.6[a]

[a] Significantly different from control.

Cd-exposed seedlings more than in controls and at a rate almost twice as high as in roots (Fig. 4B).

One function of PCs (15) and perhaps of other thiols measured in the ASTs, is binding Cd. This relationship was evaluated by determining the molar ratio of ASTs to Cd during the time course study (Fig. 4C). The ratio was high in primary roots during the early stages of the treatment (10.24 after 4 h) and decreased progressively to a plateau of approximately 2.0 after 4 d. In shoots, this ratio fluctuated much less; its peak at 4.85 occurred after 2 d of treatment, and a plateau was reached after 4 d at a slightly higher level (between 2 and 3) than in primary roots. The ratio was in the range of 2.1 to 2.9 in adventitious root tissues of Cd-exposed seedlings.

DISCUSSION

Our understanding of Cd toxicity in plants is related to the experimental design. To approach the situation in the field, intact plants exposed to environmentally realistic Cd stress are preferred to cultured cells. We decided to expose roots of intact maize seedlings to 3 μM Cd, a concentration about 8 times higher than that estimated for moderately polluted soil (30), yet much lower than the 45 to 1000 μM Cd used with cultured cells (see citations in refs. 15 and 23). Although the use of intact seedlings instead of cell cultures to study the dynamic aspects of Cd toxicity seems more appropriate physiologically, changes due to normal plant development have to be considered. For example, the changes in the morphology (*i.e.* number of leaves, appearance of adventitious roots) and the physiology (*i.e.* growth [Figs. 1 and 2] and concentrations of thiols [Fig. 3]) of the control seedlings during the duration of the time course (7 d) have to be integrated with the data from Cd-exposed plant material to understand the mechanisms of Cd toxicity.

The roots contained 60 to 67% of the Cd in the seedlings between 4 and 7 d of exposure (higher percentages earlier), and the concentration of Cd on a fresh weight basis was about 7.2-fold higher in roots than in shoots at day 7. The nature of Cd stress endured by roots and shoots differs in some ways other than only the amount of metal ions. Vögeli-Lange and Wagner (29) have shown that free Cd and Cd-binding peptides are localized in vacuoles of protoplasts isolated from tobacco leaves; current knowledge for roots is still unclear (15). An additional difference is that the entire root is immersed in a Cd solution, but the shoot acquires the metal ions through xylem translocation as for essential ions. A hyperpolarization of transroot potential due to immersion of excised maize roots in a Cd solution is known (9), but no data exist for shoot tissues. Of course, roots and shoots are interdependent, because the amount of free Cd reaching the shoot depends on the capacity of the root to retain the cytotoxic metal ions (15, 23), and the roots rely partly on shoots for the supply of reduced sulfur (19).

Separate methods were used to measure different thiols for several reasons. During HPLC analyses of acid extracts in which thiols were detected by postcolumn derivatization with Ellman's reagent (27), Cys eluted close to, and sometimes with, the solvent front. The criterion of close baseline resolution was not achieved for GSH and γEC, whereas it did apply for more hydrophobic thiols like the ASTs. Derivatization with monobromobimane allowed the quantitation of Cys, γEC, and GSH (16, 22). ASTs were quantified here as a pool of peptides appearing in maize seedlings upon exposure to Cd; they include PCs (27) and other thiols. Preliminary analyses of these other thiols indicate that most of them contain only Cys and Glu.

GSH has an essential role in sulfur nutrition of higher plants; its catabolism adds to the reduced sulfur available for protein synthesis starting from Cys (19). The endosperm of young seedlings is a major source of Cys for the scutellum, which synthesizes GSH and provides it for translocation to roots and shoots (16). Mature leaves reduce sulfate and form GSH (20), which is the major form of reduced sulfur transported over long distances in intact plants (19). The concentrations of Cys, γEC, and GSH in roots and shoots of controls changed with the development of the seedlings during the 7-d time course (Fig. 3). The concentrations of Cys and GSH declined dramatically in both roots and shoots for the 3- to 6-d-old plants. GSH was the most abundant thiol in the seedlings, although its concentration continued to decline throughout the time course. The concentrations of Cys and γEC (extremely low) were relatively stable (Fig. 3), but as roots and shoots gained weight over time (Fig. 1), the net amounts of these two thiols increased in both organs. Minor amounts of ASTs (almost exclusively PCs; ref. 27) were measured in roots and shoots of controls (Fig. 3, G and H). These thiols might be involved in metal ion homeostasis of micronutrient Zn and Cu (in our case supplied at 0.38 and 0.16

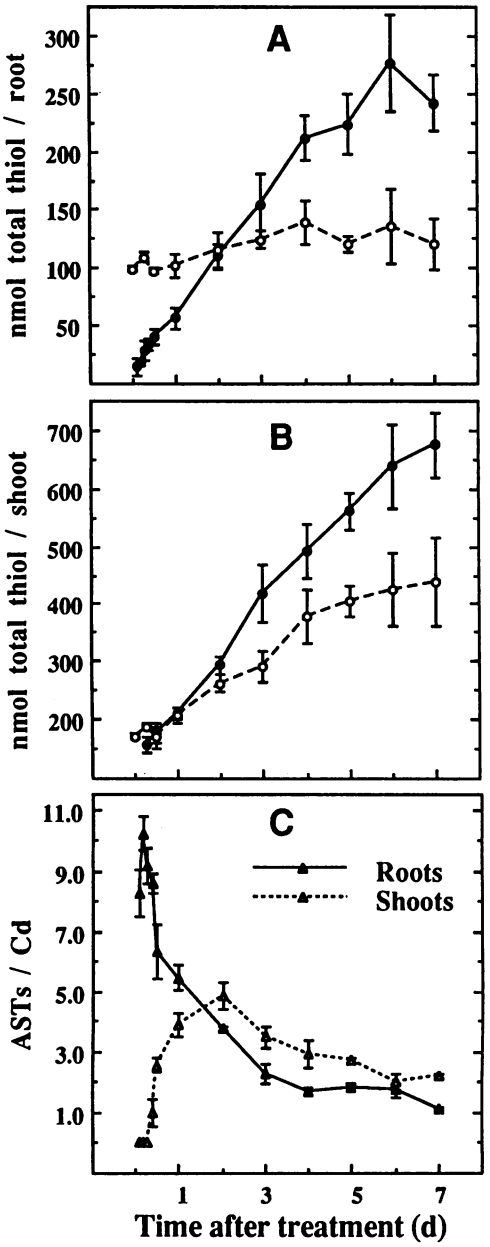

Figure 4. Amounts of total thiols per root (A), per shoot (B), and molar ratio of thiols in ASTs to Cd (C) in maize seedlings. Roots of 5-d-old seedlings were exposed to Cd concentrations of 0 (○) and 3 μM (●) for 7 d. Total thiols = Cys + γEC + GSH + ASTs. Points, Means of four replicates; vertical lines exceeding the symbols, SE.

μM, respectively) as shown in cell cultures (4). The amounts of total thiols in both roots and shoots of controls (Fig. 4) were mainly influenced by changes in GSH because the concentrations of Cys, γEC, and ASTs stayed low during the 7-d time course (Fig. 3). Expressed on a per organ basis, the amount of total thiols was constant in control roots (Fig. 4A) and increased slowly in shoots (Fig. 4B) from day 5 to day 12. The constant amount of total thiol in roots indicates a situation of equilibrium between the input (mainly GSH), consisting of phloem translocation from the endosperm and the mature leaves (16), and the output, occurring probably through the catabolism of GSH into Cys for incorporation into acid-insoluble forms of thiols (mainly proteins) (19). The slow increase of total thiols in shoots suggests a higher input than output. The input could occur through translocation of GSH from the endosperm at the early stages of development (16) and later through the reduction of sulfur within mature leaf tissues (12). The output could be a combination of GSH translocation to the root system and of thiol incorporation into proteins.

Roots of intact maize seedlings exposed to 3 μM Cd during a 2-d period reacted with a rapid and dramatic decline in the concentration of GSH and with the appearance of PCs among other ASTs (27). In addition, roots and shoots accumulated γEC soon after exposure to Cd (16). Our present data extend these measurements over a 7-d period for both roots and shoots of Cd-exposed seedlings. It is clear from Figure 3 that the effect of 3 μM Cd on the concentration of thiols in both organs was not uniform over time. In roots, the concentration of GSH decreased dramatically during the first 9 h of exposure to Cd (Fig. 3E). This decline in GSH might remove the feedback inhibition of γEC synthetase by GSH and, hence, increase the formation of γEC (7). Indeed, data in Figure 3C show accumulation of γEC after a 6-h lag phase. However, such a control mechanism cannot explain the steady increase of γEC during 6 d, while the decline of GSH in Cd-exposed roots is lower than in untreated roots from 12 h to the end of the time course (Fig. 3, C and E). Alternatively, the accumulation of γEC might be due to an elevated rate of GSH catabolism or to a differential effect of Cd on γEC synthetase and GSH synthetase, the latter being inhibited more by Cd (ref. 7; L. Bergmann, personal communication). Shoots of Cd-exposed seedlings, however, did not show a large decline of GSH, yet the concentrations of γEC increased steadily after a 2-d lag phase (Fig. 3, D and F). A full understanding of γEC accumulation in plants during Cd treatment requires further experimentation.

In cell cultures and intact plants, the early decline of GSH due to Cd treatment exactly matched the formation of PCs during a period of 2 to 9 h after starting the exposure (1, 5, 13, 21, 27). Our data extend those findings in Cd-exposed roots during the first 2 d, when the depletion of GSH (366 nmol g^{-1} fresh weight) was equivalent to the synthesis of ASTs (349 nmol g^{-1} fresh weight) (Fig. 3). However, the concentration of GSH in these Cd-exposed roots declined slowly between 2 and 7 d of exposure to Cd and could not further account for the continuous formation of ASTs (Fig. 3, E and G), unless the rate of GSH synthesis was increased. Our understanding of the formation of PCs and ASTs would, therefore, improve because of a better characterization of the rates of GSH synthesis and GSH catabolism in Cd-treated plant material. After the pool of GSH is depleted, Cd-exposed roots might use an alternative route for the continued formation of PCs and other ASTs, perhaps through the polymerization of γEC as demonstrated in fission yeast (6). No consistent decline of GSH occurred in shoots of Cd-exposed seedlings (Fig. 3F), yet significant amounts of ASTs were produced (Fig. 3H). The lack of a rapid decrease in GSH concentration usually observed in Cd-exposed plant tissues

was probably due to the high capacity of leaf tissue to reduce sulfur and produce GSH (12).

Adventitious roots of 10- to 12-d-old seedlings were also able to synthesize ASTs and to accumulate γEC upon exposure to Cd (Table I). The attachment of adventitious roots at the base of the leaves in our seedlings might explain why this root tissue reacted to Cd in ways more like shoots. As in shoots, Cd exposure did not affect the fresh weight of adventitious roots (at least after 2 d of exposure) (Fig. 1). The concentration of GSH in adventitious roots decreased early during Cd exposure as in primary roots, but afterward the variations of GSH and γEC were more like the situation in shoots (Table I, Fig. 3). Further laboratory studies might reveal whether adventitious roots can gain from a more efficient availability of reduced sulfur coming from the mature leaves than do primary roots, thus being able to deal better with cytotoxic amounts of Cd. This hypothesis should also be tested for corn grown in low to moderately polluted soils (≤0.04 μM Cd; ref. 30) to assess the role of adventitious roots in increasing the ability of older plants to deal with Cd.

A higher rate of assimilatory sulfate reduction was measured in maize seedlings exposed to 50 μM Cd during 5 d (12). Such stimulation appears rather sensitive to Cd, because exposure to only 3 μM increased the amount of total thiol in both roots (after the initial GSH decrease was overcome) and shoots (Fig. 4, A and B). If a molar ratio of thiols in ASTs to Cd of approximately 2 is considered optimal, as within the Cd-binding complex (4, 15), the amount of thiols produced in roots during the first hours of exposure to Cd was too high compared to the amount of Cd present (ratio of 10.24 after 4 h, Fig. 4C). The ratio reached a plateau in both roots (approximately 2) and shoots (between 2 and 3) after 3 to 4 d of Cd treatment, suggesting that the capacity of both organs to chelate free metal ions was now at a steady state (Fig. 4C). Ideally, the analysis of such a ratio should be performed for the Cd-binding complex after different times of exposure to Cd rather than for an acid extract in which total Cd (free + bound) is quantified. Furthermore, our use of acid extracts precluded measurements of sulfide present in Cd-binding complexes (11, 18, 25, 28). The initially high production of ASTs in excess of that required for binding Cd may reflect the abundance of the short polypeptide precursors for unrestrained synthesis of longer ones by PC synthase (6). Two complementary hypotheses are that ASTs may act as an organic store of reduced sulfur, itself abundantly available during Cd-stimulated sulfate reduction, and that some may act as the putative S carrier involved in assimilatory sulfate reduction (24, 25).

Is there a cost to a plant growing in the presence of Cd to incorporate assimilated sulfur into ASTs and Cd-binding complexes in addition to synthesis of S-containing proteins required during development? We measured the fresh weight (Fig. 1A) and the rate of root elongation (Fig. 2) to probe such a cost. After a small transitory stimulation of the rate of elongation, Cd-exposed roots elongated more slowly than controls for 2.5 d. Most of the elongation occurs within 10 mm of the root apex and the contribution of the fresh weight of this zone to the fresh weight of the entire root system is probably too small to be detected as an early effect of Cd toxicity (Fig. 1A). As discussed above, the stable amount of total thiols in control roots (Fig. 4A) results from a balance between the input of GSH and the output through the incorporation of Cys into proteins. Exposing roots to Cd perturbed this equilibrium (Figs. 3 and 4A); therefore, less reduced sulfur was available for the growth of the root as shown by a decreased rate of elongation (Fig. 2), part of which might also be due to other toxic effects of Cd. The rate of root elongation of Cd-exposed seedlings began to recover between 2.5 and 4.5 d and was even higher than in controls during the last 2 d of the time course (Fig. 2), whereas the fresh weight was reduced from day 4 onward (Fig. 1A). The timing of full recovery of root elongation (from day 4.5, Fig. 2) corresponds to the stabilization of the molar ratio of ASTs to Cd at approximately 2, suggesting that enough Cd is chelated to permit the root to elongate in a new state of equilibrium. However, a certain developmental cost was paid by the Cd-exposed roots, as shown by their reduced fresh weight (Fig. 1A). Primary roots of 5-d-old seedlings exposed to 3 μM Cd during a 7-d period appear to adapt their metabolism by reducing growth (*i.e.* fresh weight gain) and switching the supply of reduced sulfur to the mechanism of Cd chelation. Because the primary root system has a limited life span, the ability of older plants to survive in an environment containing Cd may come to rely on the capacity of adventitious roots to adapt and chelate Cd.

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