Plant Physiol. (1992) 99, 428-433
0032-0889/92/99/0428/06/$01.00/0

Received for publication September 21, 1991
Accepted December 11, 1991

Effect of Cadmium on γ-Glutamylcysteine Synthesis in Maize Seedlings[1]

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ABSTRACT

Cysteine, γ-glutamylcysteine, and glutathione and the extractable activity of the enzymes of glutathione biosynthesis, γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3), were measured in roots and leaves of maize seedlings (*Zea mays* L. cv LG 9) exposed to $CdCl_2$ concentrations up to 200 micromolar. At 50 micromolar Cd^{2+}, γ-glutamylcysteine contents increased continuously during 4 days up to 21-fold and eightfold of the control in roots and leaves, respectively. Even at 0.5 micromolar Cd^{2+}, the concentration of γ-glutamylcysteine in the roots was significantly higher than in the control. At 5 micromolar and higher Cd^{2+} concentrations, a significant increase in γ-glutamylcysteine synthetase activity was measured in the roots, whereas in the leaves this enzyme activity was enhanced only at 200 micromolar Cd^{2+}. Labeling of isolated roots with [^{35}S]sulfate showed that both sulfate assimilation and glutathione synthesis were increased by Cd. The accumulation of γ-glutamylcysteine in the roots did not affect the root exudation rate of this compound. Our results indicate that maize roots are at least in part autonomous in providing the additional thiols required for phytochelatin synthesis induced by Cd.

In plants exposed to heavy metals, the formation of metal-binding polypeptides with the general structure (γ-glutamyl-cysteine)$_n$glycine, n = 2 to 11, known as phytochelatins is induced (18, 25). Because of the presence of γ-glutamic acid linkages in the peptide chain, phytochelatins cannot be primary gene products. An enzyme synthesizing phytochelatins has been found in *Silene cucubalus* cell cultures (6, 12) and in fission yeast (30). Phytochelatin synthase or γEC[2] dipeptidyl transpeptidase forms the peptide chain by the addition of γEC moieties from GSH to an acceptor GSH molecule. Consistent with this, the GSH content decreases, at least initially, because of Cd treatment in cell cultures (3, 23), roots (19, 21, 28), and both roots and shoots (17).

GSH is synthesized in two steps by the action of γEC synthetase (EC 6.3.2.2), which joins Cys with glutamic acid, followed by GSH synthetase (EC 6.3.2.3), which adds glycine to γEC (8, 9).

Although the root is the organ primarily affected by heavy metals, no information is available about the *in vivo* capacity of roots to synthesize GSH or about the response of γEC synthetase activity in plants treated with heavy metals.

Yeast mutants lacking a sufficient GSH synthetic capacity are hypersensitive to Cd (5, 14). The suppression of GSH formation and thus phytochelatin synthesis by the application of buthionine sulfoximine, which inhibits the activity of γEC synthetase, enhances the toxic effect of Cd (10, 13, 20, 21, 26), silver (7), and copper ions (22). These observations indicate the role of phytochelatins for the detoxification of heavy metal ions (3, 11, 29) and the particular importance of γEC synthesis in plants exposed to heavy metals.

In the present study, we examined the pathway leading to GSH in plants exposed to Cd with special emphasis on γEC synthetase activity and γEC contents in roots, and we determined the effect of Cd on the rate of *in vivo* sulfate assimilation and GSH formation in isolated maize roots.

MATERIALS AND METHODS

Plant Material and Treatment

Maize kernels (*Zea mays* L. cv LG 9; Limagrain, Ennezat, France) were soaked for 1 d in aerated water at room temperature, germinated between several layers of damp paper in the dark at 23 to 25°C for 2 d, and placed in pots with 320 mL of nutrient solution (16). These pots contained 22 seedlings each for the root exudate determination, 16 for the 4-d kinetic experiments, and 10 for the growth measurements and estimations performed after a 4-d treatment. All plants were cultivated in continuous light (70 $\mu E\ m^{-2}\ s^{-1}$, provided by three TL 40 W/34 from Philips and one L 36/W 77 Fluora from Osram) at 23 to 25°C and 60 to 65% RH. Three days after the plants were transferred to the cultivation pots, Cd was added to the nutrient solution at the various concentrations as $CdCl_2$.

For [^{35}S]sulfate labeling, the roots of six plants each of controls and Cd treatments were cut about 5 mm below the kernels and placed with the cut surface above the liquid level in 30 mL of nutrient solution with or without 50 μM Cd^{2+}, containing 75 instead of 750 μM sulfate and 1 mCi of [^{35}S] sulfate. After 4 h, the roots were rinsed, kept in ice-cooled water for 10 min, and rinsed again before extraction.

To collect root exudate, the shoots of at least 20 plants per treatment were cut off about 10 mm above the position of the kernels. The cut surfaces were rinsed with double-distilled water, excess moisture was removed using damp paper, and the plants were placed in a box with 100% RH. After 30 and 60 min, the fluid exuding from the cut surfaces was collected with a pipette and kept on ice until assayed for thiols.

[1] Supported in part by the Swiss National Science Foundation and by the "Europäischer Förderpreis" of the Körber Foundation.

[2] Abbreviations: γEC, γ-glutamylcysteine; CHES, 2-(cyclohexylamino)-ethanesulfonic acid.

Preparation of Extracts

Root systems were rinsed extensively with tap water and cut off about 5 mm below the kernel. Second leaves were excised together with their leaf sheath. Plant material of four to six plants was ground in an ice-cooled glass homogenizer, and the homogenate was made cell free by filtration through one layer of 100% viscose fleece (Milette, Migros, Switzerland). For the thiol measurement, one part of plant material was extracted with 10 parts (w/v) of 0.1 N HCl containing 1 mM Na-EDTA except for ^{35}S-radiolabeled roots, for which the ratio was 1:5 (w/v). For the measurement of γEC synthetase, one part of roots or leaves was extracted with three or five parts (w/v), respectively, of 0.1 M Tris-HCl containing 5 mM EDTA (pH 8.0). For the GSH synthetase assay, one part of plant material was extracted in five or 10 parts (w/v) of 0.1 M Tris-HCl containing 10 mM $MgCl_2$, 1 mM EDTA (pH 7.5) for roots and leaves, respectively.

Determination of Cys, γEC, and GSH

Thiols were separated and quantified by reverse-phase HPLC after reduction with $NaBH_4$ and derivatization with monobromobimane (15, 24). Extracts were centrifuged for 30 min at 30,000*g* and 4°C. To 400 μL of supernatant, 600 μL of 0.2 M CHES (pH 9.3) and 100 μL of a freshly prepared 40 mM $NaBH_4$ solution were added. The blank was extraction medium. This reduction step was modified for root exudates to accommodate the small volume of exudate with a pH value different from extracts: 100 μL of exudate (diluted with 1 mM HCl if smaller amounts obtained) was mixed with 200 μL of 50 mM CHES (pH 9.0) and 100 μL of a freshly prepared 20 mM $NaBH_4$ solution. The mixture was kept on ice for 20 min. For derivatization, 330 μL of this mixture was added to 15 μL of 15 mM monobromobimane dissolved in acetonitrile and kept in the dark at room temperature for 15 min. Derivatization was terminated by the addition of 250 μL of 5% (v/v) acetic acid. The samples were centrifuged for 10 min at 16,000*g* and 4°C, and the undiluted supernatant was used for the measurement of Cys and γEC. A 20-fold dilution with 2.5% (v/v) acetic acid was used for the measurement of GSH. The samples were analyzed according to the method of Schupp and Rennenberg (24) on a System Gold HPLC system (Beckman, Basel, Switzerland) with a Nucleosil 100–5 C_{18} column (4.0 × 250 mm i.d., 5 μm particle size; Macherey-Nagel, Oensingen, Switzerland) and an SFM 25 fluorescence detector (Kontron, Zurich, Switzerland).

Measurement of ^{35}S-Label in Thiols and Sulfate

To obtain sufficient radioactivity for detection after HPLC separation, more concentrated samples were prepared using 55 μL of 20% acetic acid to terminate the derivatization step. A 100-μL aliquot of each sample was separated as mentioned for inactive thiols, and fractions of 23 drops each were collected in scintillation vials between retention times of 5 and 17 min. Ultima Gold XR scintillation cocktail (2 mL; Packard, Zurich, Switzerland) was added per fraction, and the radioactivity was counted in a Betamatic V liquid scintillation counter (Kontron, Zurich, Switzerland). To quantify thiols from the same extract as radiolabel, the samples were diluted with 2.5% (v/v) acetic acid to adjust to the detection range of the fluorescent measurement. For the quantification of [^{35}S] sulfate, a 500-μL aliquot of the eluate collected between 0 and 5 min was counted as described for thiols.

Enzyme Assays

For the determination of γEC synthetase activity, a modification of the assay described by Hell and Bergmann (9) was used. The extract was centrifuged for 10 min at 30,000*g* and 4°C. A 650-μL aliquot of the supernatant was desalted according to the method of Feller *et al.* (4) using 6 mL of Sephadex G-25 (Pharmacia, Dübendorf, Switzerland) equilibrated with extraction buffer. Before the extract was loaded, the column (13 × 56 mm) held in a conical tube was centrifuged for 3 min at 250*g*, followed by 3 min at 400*g*, and the excess buffer was removed from the tube. After the extract was loaded, the column was kept on ice for 10 min and then centrifuged as previously indicated, the eluate being used for the assays. Desalting of the extract excluded inhibitory concentrations of Cd and/or GSH in the incubation assays. The reaction mixture contained, in a total volume of 500 μL, 0.1 M Hepes-NaOH (pH 8.0), 40 mM $MgCl_2$, 30 mM Na-L-glutamate, 0.8 mM L-Cys, 0.4 mM DTE, 7 mM ATP, and 250 μL of extract containing 60 to 260 μg of protein. Incubation was for 45 min at 37°C. For derivatization, 50-μL aliquots of the assay mixture were transferred to 200 μL of 50 mM CHES (pH 9.0) and 15 μL of 15 mM monobromobimane dissolved in acetonitrile. After 15 min at room temperature in the dark, derivatization was terminated by the addition of 700 μL of 5% (v/v) acetic acid. Blank values were obtained from nonincubated reaction mixtures. γEC was separated from Cys by HPLC as mentioned for the thiol measurement in extracts, but with 10% methanol applied between 0 and 8 min retention time, and then methanol was increased to 16% within 8 min.

GSH synthetase activity was measured according to the method of Hell and Bergmann (8). The extract was centrifuged for 20 min at 30,000*g* and 4°C and desalted as described for the γEC synthetase measurement. The assay system contained, in a final volume of 500 μL, 90 mM Tris-HCl (pH 8.4), 20 mM Mg_2Cl, 45 mM KCl, 1 mM glycine, 0.5 mM γEC, 5 mM phospho*enol*pyruvate, 5 units of pyruvate kinase as an ATP-regenerating system, 4.5 mM DTE, and 200 μL of extract containing 40 to 140 μg of protein. After incubation for 60 min at 37°C, 25-μL aliquots were derivatized in 200 μL of 50 mM CHES (pH 8.4) and 20 μL of 15 mM monobromobimane dissolved in acetonitrile. Derivatization was carried out for 15 min at room temperature in the dark and stopped by the addition of 1.0 mL of 5% (v/v) acetic acid. Blank values were obtained from nonincubated assay mixture. GSH was separated from γEC and DTE as described for the thiol measurement in extracts.

Protein Determination

The protein content of the extracts was measured according to the method of Bradford (2) with BSA as the standard.

Chemicals

Monobromobimane was obtained from Calbiochem (La Jolla, CA), γEC was from Nacalai Tesque (Kyoto, Japan), [^{35}S]sulfate came from the Radiochemical Centre (Amersham, United Kingdom), and all other chemicals were purchased from Fluka (Buchs, Switzerland).

RESULTS

After 4 d with 50 or 200 μM Cd^{2+}, growth of roots and shoots of maize seedlings was significantly reduced (Fig. 1). At 200 μM Cd^{2+}, roots did not grow any more on a fresh weight basis, whereas the increase in fresh weight of shoots was still 32% of the control value. Cd concentrations of 0.5 and 5 μM affected neither root nor shoot growth significantly.

With 50 μM Cd^{2+}, three different types of changes were measured for Cys, γEC, and GSH in a typical experiment (Fig. 2): (a) the Cys contents increased to 200% of the control value in roots and leaves with a large increase on the second day with Cd^{2+} (top panels); (b) in roots, the γEC content increased continuously from the beginning; in leaves, the increase started after 1 d with Cd, and by the end of the experiment, concentrations that were 21-fold and eightfold of the control were reached in roots and leaves, respectively (middle panels); and (c) in roots GSH decreased considerably during the first day with Cd reaching a level between 40 and 65% of the controls for the remaining time of the experiment;

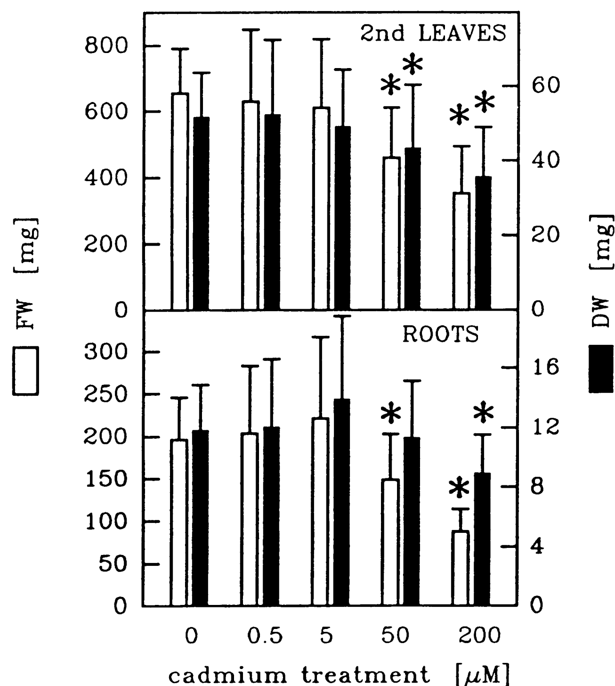

Figure 1. Fresh weight (FW) and dry weight (DW) of roots and shoots of 10-d-old maize seedlings after 4 d treatment with Cd. Mean values and SD were calculated from samples of 16 plants each. The values on day 6, before Cd addition, were 197.3 and 18.1 mg and 90.6 and 5.6 mg for shoot and root fresh and dry weight, respectively. Asterisks indicate a significant difference from the controls (Wilcoxon, $\alpha \leq 0.05$).

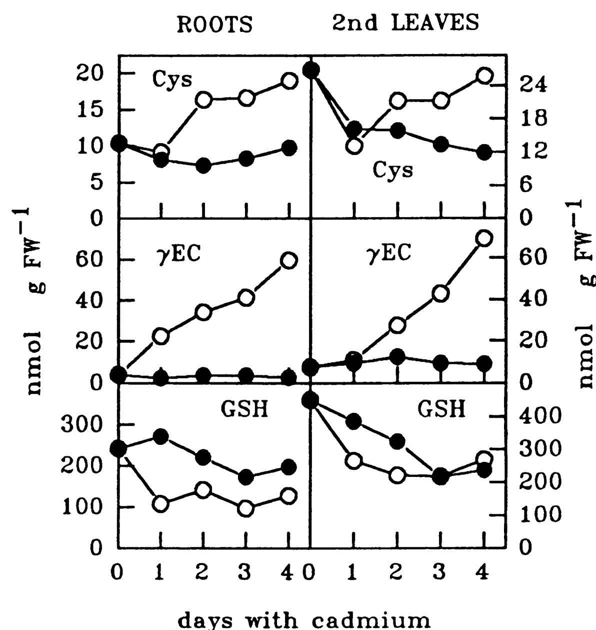

Figure 2. Contents of Cys, γEC, and GSH in roots and second leaves of 6- to 10-d-old maize seedlings grown without Cd (●) or exposed to 50 μM Cd^{2+} (○) during the experiment. FW, Fresh weight.

in leaves of controls, GSH decreased slower than in Cd-treated plants but reached the same concentration after 3 d (bottom panels).

In view of the considerable increase in γEC of roots and leaves, the extractable activity of γEC synthetase was measured. At 50 μM Cd^{2+}, the activity in roots increased more than twofold in a typical experiment, whereas, in leaves, this enzyme activity of both treated and nontreated plants declined to 35% of the initial value during the first day of the experiment (Fig. 3). Only at 200 μM Cd^{2+}, a level of 150 to 190% of the control value of γEC synthetase activity was determined in leaves (data not shown). The action of various Cd concentrations on γEC content and γEC synthetase activity from roots is presented in Figure 4. A significant increase was measured at ≥5 μM Cd^{2+} treatments for the enzyme activity and as low as 0.5 μM Cd^{2+} for γEC. At 200 μM Cd^{2+}, no more enzyme activity than at 50 μM was induced, but the content per gram of fresh weight in γEC increased even more to a level that was 36 times higher than that of the control.

For comparison, the extractable activity of GSH synthetase was determined. Within 4 d at 50 μM Cd^{2+}, this enzyme activity showed maximal effects with values of 1.7 and 0.8 nmol GSH produced per milligram of protein per minute in roots (140% of control) and in leaves (160% of control), respectively (mean values of three independent experiments, data not shown).

To study the effect of Cd on *in vivo* synthesis of γEC and other thiol compounds in roots, [^{35}S]sulfate was fed to the isolated roots. Although the ^{35}S label in sulfate was lower because of Cd, the incorporation of radioactivity from [^{35}S]sulfate into Cys, γEC, and GSH was markedly enhanced in roots from Cd-treated plants (Fig. 5). The specific radioactivity of γEC was higher in control roots than that of its precursor

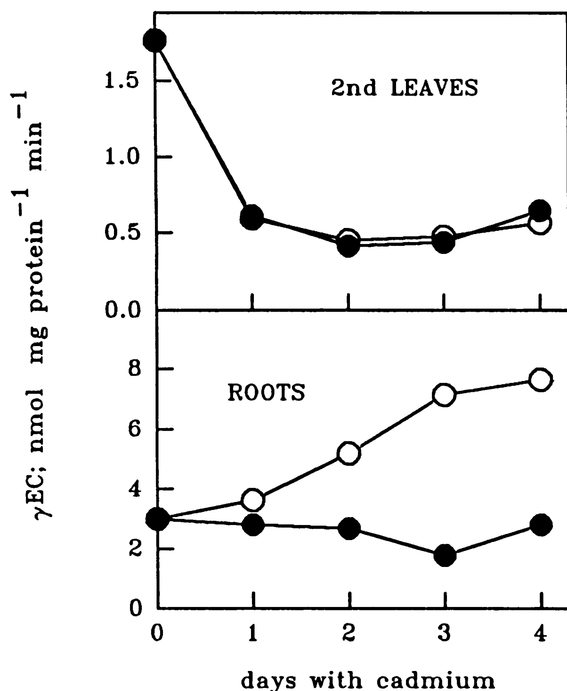

Figure 3. Extractable activity of γEC synthetase in second leaves and roots of 6- to 10-d-old maize seedlings grown without Cd (●) or exposed to 50 μM Cd^{2+} during the experiment (○).

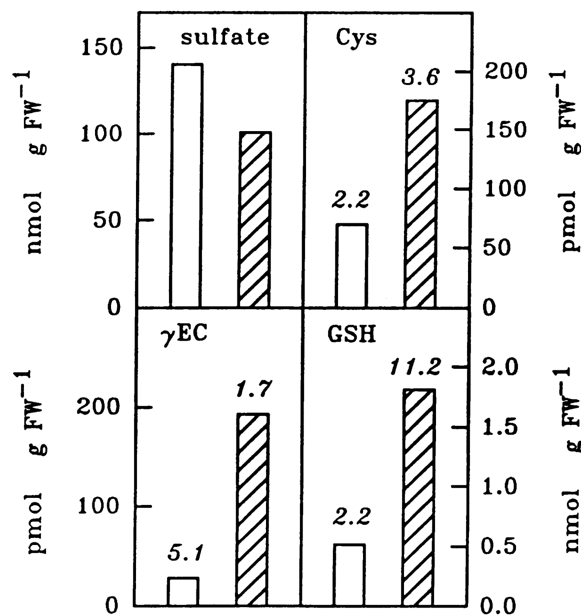

Figure 5. ^{35}S-radiolabeled compounds in roots of 8-d-old maize seedlings. Radioactivity as [^{35}S]sulfate was supplied during 4 h to the isolated roots of plants without (□) or with a 2-d pretreatment with 50 μM Cd^{2+} (▨). Italic numbers indicate the portion of labeled thiols in relation to the total of the individual thiols in curies per mole. Mean values of two independent experiments are presented. FW, Fresh weight.

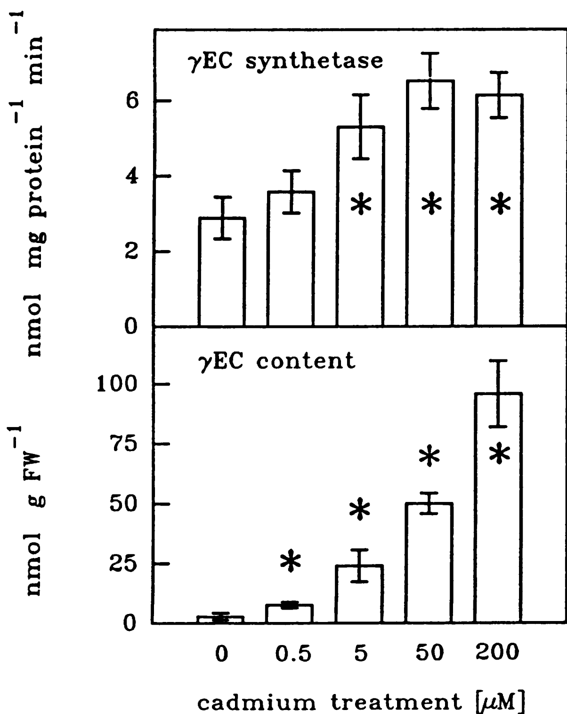

Figure 4. Extractable activity of γEC synthetase and γEC content in roots of 10-d-old maize seedlings cultivated with different Cd concentrations for the last 4 d. Mean values ± SD of five independent experiments are presented. Values with an asterisk differ significantly from the control (Wilcoxon, $\alpha \leq 0.05$). FW, Fresh weight.

Cys, indicating one or more metabolic pool(s) of Cys with a low turnover. In the roots of Cd-treated plants, GSH had a higher specific activity than γEC. This demonstrates that Cd^{2+} not only induced an increase in γEC but also one or more metabolic pool(s) of γEC with a low turnover were formed or expanded.

The large accumulation of γEC in the roots caused by Cd suggested that, under these conditions, this thiol may be transported from roots to shoots. To test this hypothesis, the thiols in the exudates of roots with or without kernels were measured. Cd caused significantly smaller fluid exudation rates (mean values ± SD of six independent experiments, Wilcoxon $\alpha \leq 0.05$): 7.7 ± 2.7 μL of root exudate was collected per root per h for controls; 7.1 ± 2.6 μL for roots without kernels; 3.8 ± 0.8 μL for roots with kernels after 2 d with 5 μM Cd^{2+}; and 3.8 ± 0.7 μL for roots without kernels but with Cd^{2+} (data not shown). γEC was detected in all exudates, but Cd had no effect on its exudation rate (Fig. 6) because the concentration of this thiol was higher in the exudates of Cd-treated plants. Less Cys was exuded because of Cd in the presence or absence of the kernels. A smaller amount of GSH was present in root exudates only as a result of kernel removal.

DISCUSSION

Our results show an enormous increase in γEC both in roots and shoots of Cd-treated maize seedlings. In roots, this increase can be explained by the higher level of γEC synthetase activity and the higher contents of Cys, which may increase the rate of γEC formation (9). Additionally, the lower

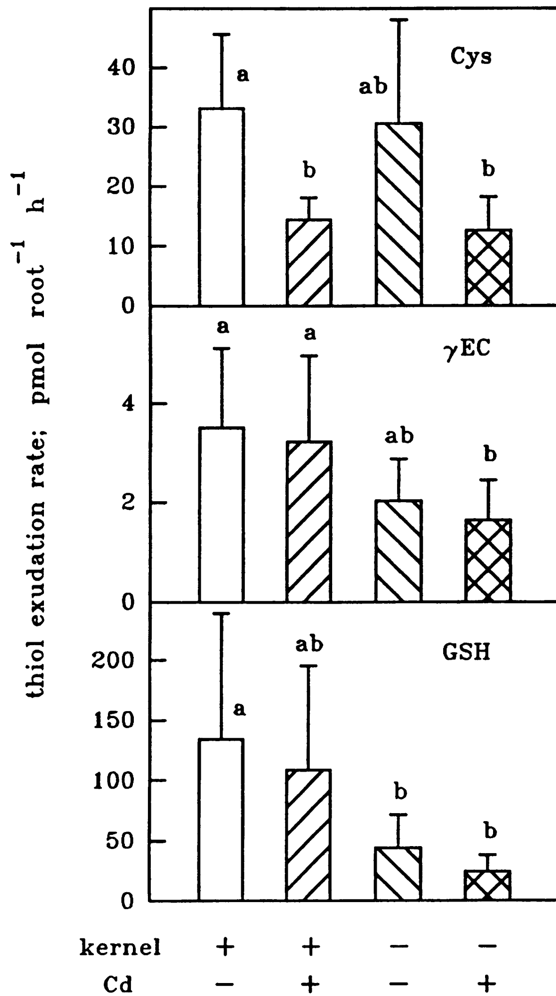

Figure 6. Cys, γEC, and GSH in exudates from roots of 8-d-old maize seedlings collected during the first hour after shoot removal in the presence or absence of the kernels. Roots from plants cultivated without or with 5 μM Cd^{2+} during the last 2 d were used. Mean values ± SD of six independent experiments are shown. Exudation rates of individual thiols with different letters are significantly different (Wilcoxon, $\alpha \leq 0.05$).

levels of GSH may eliminate a possible feedback inhibition of γEC synthetase (9). Indeed, the [^{35}S]sulfate-feeding experiment with isolated roots of Cd-treated plants showed an enhanced radioactive labeling of γEC, even though more γEC was used at the same time for GSH synthesis compared with the control, demonstrating an increased formation of γEC by the roots. These findings are supported by the fact that GSH is subsequently used as a substrate for the synthesis of phytochelatins (6), the major thiol sink in Cd-treated plants (28). At 200 μM Cd^{2+}, two times more γEC accumulated in the roots than at 50 μM Cd^{2+}, but no further increase in γEC activity was measured. This effect may be explained in part by the higher dry weight to fresh weight ratio at 200 μM Cd^{2+} and in part by the larger accumulation of the substrate Cys at this Cd concentration than at 50 μM Cd^{2+} (data not shown).

In the leaves, the increased availability of Cys might be the main reason for the accumulation of γEC at 50 μM Cd^{2+}, because γEC started to accumulate only in the presence of increased Cys levels. As mentioned for roots, γEC synthesis may be enhanced, in addition, by a smaller feedback inhibition of γEC synthetase by GSH, which is at a lower level because of Cd treatment. The fact that 50 μM Cd^{2+} induced a similar accumulation of γEC both in leaves and roots but only an increase in γEC synthetase activity in roots can be explained by a lower consumption of γEC for phytochelatin synthesis in leaves compared to roots (16). A higher extractable activity of γEC synthetase was measured in the leaves only at 200 μM Cd^{2+}. It may be required to enhance the effect of increased substrate availability at high Cd doses. The rate of γEC exuded from Cd-treated roots was not different from controls, making it unlikely that transport of γEC from roots to shoots induced the accumulation in the leaves. Net flux of thiols from the roots to the shoots might not occur because GSH is probably transported to the roots also; up to 1 mM GSH was present in phloem exudates from *Cucurbita* species (1). Furthermore, the estimated flux of GSH from seedlings fed with ^{35}S-Cys by injection to the endosperm was not increased as a result of Cd treatment (19).

Additional support for the importance of sufficient γEC synthesis in plants exposed to Cd comes from the observation that a higher activity of γEC synthetase was measured in Cd-resistant than in Cd-sensitive tomato cells (27).

The enhanced formation of GSH in maize roots treated with Cd as demonstrated by the present ^{35}S-labeling data may be caused both by a larger availability of the substrate γEC and by the higher amount of GSH synthetase. The fact that pea plants produce more additional thiols because of Cd treatment (21) than maize may be explained by the larger increase in GSH synthesis as a result of both an up to sixfold increase in extractable activity of GSH synthetase (21) and an accumulation of γEC (our unpublished results) similar to the one presented here for maize.

Taken together, our results demonstrate a contribution of maize roots to thiol synthesis and the importance of γEC synthetase for covering the increased demand for thiols induced by Cd. Furthermore, the results indicate that, with Cd, the GSH synthetase reaction is the rate-limiting step of GSH synthesis, probably due to a higher sensitivity of GSH synthetase than γEC synthetase to Cd as reported for the partially purified enzymes from *Petrosilenum crispum* (19).

ACKNOWLEDGMENT

We thank Dr. Mary Kavanagh for improving the style of the manuscript.

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