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Hydroxymethyl-Phytochelatins [(γ-Glutamylcysteine)$_n$-Serine] Are Metal-Induced Peptides of the Poaceae[1]

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Exposure of several species of the family Poaceae to cadmium results in the formation of metal-induced peptides of the general structure (γ-Glu-Cys)$_n$-Ser (n = 2–4). They are assumed to be formed from hydroxymethyl-glutathione (γ-Glu-Cys-Ser) and are termed hydroxymethyl-phytochelatins (hm-PCs) in analogy to the homo-phytochelatins [(γ-Glu-Cys)$_n$-β-Ala], discovered in legumes, and the phytochelatins [PCs, (γ-Glu-Cys)$_n$-Gly] found in most other plants and many fungi. The hm-PCs were isolated from the roots of cadmium-exposed rice (*Oryza sativa* L. cv Strella), and their structure was confirmed by amino acid analysis after total and enzymic hydrolysis and by tandem mass spectrometry. The hm-PCs probably play a significant role in heavy metal detoxication in rice. In addition to this new form of γ-Glu-Cys (γEC) peptide, PCs and γEC peptides without C-terminal Ser or Gly are found. All γEC peptides are synthesized without delay after incubation of rice plants in 100 μM $CdCl_2$ in the roots as well as in the shoots. Incubation times exceeding 24 h or higher concentrations of cadmium result in a selective enrichment of γEC peptides with higher chain length and an increased ratio of PCs to hm-PCs. γEC peptide synthesis is accompanied by a decrease of the glutathione content and an increase of the hydroxymethyl-glutathione content in roots and shoots of rice plants.

Phytotoxic amounts of metals, including Cd, Zn, Cu, and Pb, are occasionally found in soils under natural conditions, but they originate more frequently from industrial and agricultural activities. In response to these elements, higher plants, algae, and some fungi synthesize peptides consisting of repeating units of γ-glutamylcysteine followed by a single C-terminal Gly (Gekeler et al., 1988, 1989; Rauser, 1990; Steffens, 1990). The number of repeating units ranges from 2 to 11 and varies with the concentration and duration of metal exposure (Hayashi and Nakagawa, 1988; Tukendorf and Rauser, 1990). These peptides are named PCs (Grill et al., 1985), poly(γ-glutamylcysteinyl)glycines, or (γEC)$_n$Gs (Robinson and Jackson, 1986), cadystins (Kondo et al., 1984), γ-glutamyl peptides (Reese et al., 1988), and γEC peptides (Dameron et al., 1989b).

The (γEC)$_n$G peptides bind metal ions in a metal-cysteinyl thiolate cluster composed of multiple peptides that are heterogeneous in length and metal ions (Reese et al., 1988). Incorporation of acid-labile S^{2-} into the Cd-peptide complexes increases the metal-binding affinity and Cd stoichiometry of the peptides (Reese and Winge, 1988) and is found in complexes of higher apparent molecular masses (Barbas et al., 1992). A complex isolated from *Candida glabrata* has a S:Cd ratio of 0.7 and contains a 2.0-nm-diameter CdS crystallite core of about 80 CdS units, coated with nearly 30 peptides (Dameron et al., 1989a).

The biosynthesis of the (γEC)$_n$G peptides in plants is catalyzed by the metal-activated and constitutive enzyme PC synthase, which transfers the glutamylcysteine moiety of GSH to an acceptor GSH molecule or to an existing (γEC)$_n$G peptide, yielding (γEC)$_{n+1}$G (Grill et al., 1989; Loeffler et al., 1989). An alternative pathway found in the fission yeast is γ-Glu-Cys polymerization from (γEC)$_n$ and GSH to (γEC)$_{n+1}$, followed by Gly addition with GSH synthetase (Hayashi et al., 1991). In this yeast, peptides of the structure (γEC)$_n$ are constituents of the metal-binding complexes (Mehra and Winge, 1988). They are termed desGly-PCs and are also found in other organisms (Bernhard and Kägi, 1987; Barbas et al., 1992). Exposure of several species of the order Fabales to Cd results in the formation of peptides of the general structure (γ-Glu-Cys)$_n$-β-Ala (Grill et al., 1986). They are assumed to be formed from h-GSH, which partly or completely replaces GSH in these plants (Klapheck, 1988), and are termed h-PCs. The common structural element of the h-PCs, the desGly-PCs, and the PCs is the γEC unit; therefore, the general name γEC peptide will be used for them in this report.

In Cd-binding complexes isolated from roots of *Agrostis gigantea*, some peptides containing Ser are found (Rauser et al., 1986, 1988), but their primary structure has not been elucidated. Since these peptides have equimolar quantities of Glu and Cys in addition to Ser, but no Gly, a structural homology to PCs seems possible. We now provide evidence that when exposed to heavy metals, in addition to PCs and desGly-PCs, γEC peptides of the general structure (γ-Glu-Cys)$_n$-Ser are synthesized in plants of the family Poaceae. These Ser-containing peptides are structurally related to the tripeptide γ-Glu-Cys-Ser, which is present in addition to GSH in many species of this family (Klapheck et al., 1992) and which has been termed hm-GSH (Bergmann and Rennenberg, 1993). In analogy to the h-PCs these peptides, therefore, are named hm-PCs. We determined the effect of Cd on the dynamics of appearance of all three γEC peptide

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Abbreviations: desGly-PC, desGly-phytochelatin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); h-GSH, homo-glutathione; h-PC, homo-phytochelatin; hm-GSH, hydroxymethyl-glutathione; hm-PC, hydroxymethyl-phytochelatin; PC, phytochelatin.

isoforms and on the changes in the content of the putative precursors GSH and hm-GSH.

MATERIALS AND METHODS

Plant Material

Seeds of rice (*Oryza sativa* L. cv Strella) were purchased from Bertone Sementi (Ferruggia, Italy). *Triticum aestivum* L. cv Star, *Avena sativa* L. cv Regent, and *Secale cereale* L. cv Kustro were from Lochow-Petkus GmbH (Bergen, Germany). *Panicum violaceum* L. and *Pennisetum setaceum* Hochst. were from Walz (Stuttgart, Germany). *Zea mays* L. cv Badischer Landmais and *Phleum pratense* L. were from Schmitz and Laux (Hilden, Germany).

Seeds of rice were soaked overnight in aerated water at room temperature. Plants were grown hydroponically in Hoagland solution at 30°C and 100% RH in the dark for 2 to 3 d using 10- × 20-cm plastic screens placed above plastic pots of 10 cm height. The pots were then transferred to a growth cabinet at 27°C by day, 24°C by night, 70% RH, a light period of 16 h, and a light intensity of 130 μE m^{-2} s^{-1} at the top of the plants. Plants (6–7 d old) were exposed to $CdCl_2$ or the acetate salts of Zn^{2+} and Pb^{2+} in fresh nutrient solution. All other plants were grown hydroponically in Hoagland solution in the growth cabinet for 5 d before exposure to 250 μM $CdCl_2$ for 3 d.

Analysis of γEC Peptides and Thiols

The roots were extensively washed with deionized water and all roots were cut off about 1 cm below the seed. The whole shoot was cut off and the coleoptile was removed. Roots or shoots were pulverized in liquid N_2 and homogenized with 0.1 N HCl, using one part plant material and four parts (w/v) HCl. The extracts were centrifuged (12,000*g*, 3 min) and analyzed at once or stored at −20°C.

The GSH and hm-GSH contents were determined after reduction with DTE and derivatization with monobromobimane by HPLC separation on RP_{18} columns and detection by fluorescence (Klapheck, 1988). γEC peptides were analyzed by HPLC using the DTNB postcolumn reaction (Grill et al., 1986). To 600 μL of the extracts 150 μL of 0.5 M Tris/HCl, 10 mM DTE (pH 8.0) were added, and the pH was adjusted to 8.0 with 1 N KOH. Reduction was terminated by addition of TFA to a 5% (v/v) final concentration after 30 min of incubation at room temperature, and the precipitate was removed by centrifugation. Samples (50 μL) were separated on an RP_{18} column (4.6 × 250 mm, Hypersil, 5-μm corn size) with a linear gradient of 0 to 36% (v/v) methanol in 0.05% (v/v) TFA in 36 min at a flow rate of 1 mL min^{-1}. To the column effluent a 75 μM DTNB solution in 50 mM K-phosphate buffer (pH 8) was mixed at 1 mL min^{-1}. The sulfhydryl-containing compounds were detected at 410 nm after passing a reaction loop of 0.5 mm × 3 m. The cellular concentrations are reported as γEC equivalents that are based on HPLC peak areas of γEC peptides quantified with GSH as a standard. γEC peptide and thiol contents were related to the fresh weight of the plant material.

Purification of γEC Peptides and Structure Analysis

The roots of rice plants exposed to 100 μM $CdCl_2$ for 2 to 5 d were washed thoroughly and homogenized in liquid N_2. The frozen powder was added to three parts (w/v) of 0.1 N HCl, homogenized once more, and centrifuged at 40,000*g* for 10 min. To the supernatant corresponding to 10 g fresh weight of roots, 6 mL of 0.5 M Tris, 30 mM DTE, and 2 mM EDTA were added, and the pH was adjusted to 8.0 with KOH. The solution was centrifuged (40,000*g*, 10 min), and the supernatant was diluted with an equal volume of distilled water. The solution was loaded on an anion-exchange resin (QAE Sephadex A-25, 1.5 × 2 cm; Pharmacia, Uppsala, Sweden). The column was washed with 15 mL of 50 mM K-acetate buffer (pH 5.5), followed by 10 mL of water. Acid peptides were eluted with 0.1 N HCl, and fractions of 2 mL were collected. All fractions were analyzed for γEC peptide content by HPLC using the DTNB postcolumn reaction. The fraction with the highest γEC peptide concentration was used for the isolation of the individual isopeptides by reverse-phase HPLC using the same conditions as given above with the exception that the postcolumn derivatization was omitted. Components were detected in the eluant by A_{220}, and peaks were collected individually. To collect adequate quantities of each unique γEC peptide, peak fractions of four to seven runs were pooled.

Each sample was subjected to performic acid oxidation, complete acid hydrolysis, *o*-phthalaldehyde derivatization, and amino acid analysis (Klapheck, 1988). For the enzymic hydrolysis with carboxypeptidase A from bovine pancreas (E.C. 3.4.17.1, Sigma) (Klapheck et al., 1992) HPLC eluant fractions containing only one isoform of γEC peptide were lyophilized and dissolved in enzyme buffer. Release of Ser or Gly was determined by amino acid analysis, and release of $(\gamma EC)_n$ was analyzed by the DTNB postcolumn reaction HPLC method.

Positive fast atom bombardment tandem MS was done using a Finnigan H-SQ30 system (Bremen, Germany) with a BEqQ configuration. An aliquot of the lyophilized $(\gamma$-Glu-Cys$)_3$-Ser peptide was dissolved in distilled water, and thioglycerol was added as matrix. The sample was irradiated with a beam of 8-kV xenon ions. $(M+H)^+$ ions were selected in the first sector of the double-focus system and subjected to collisionally activated dissociation with argon in the first quadrupole. Daughter ions were scanned in the second quadrupole.

RESULTS

Elucidation of hm-PC Structure

Exposure of the roots of rice plants to Cd and subsequent analysis of the crude extract by HPLC with thiol-specific DTNB postcolumn reaction demonstrates that, in addition to the PCs with $n = 2$, 3, and 4 γEC units found in most plants, several other thiols are formed (Fig. 1). The presence of hm-GSH in addition to GSH in these plants (Klapheck et al., 1992), as well as the HPLC partition pattern, suggests that part of these additional Cd-induced thiols are γEC peptides with C-terminal Ser.

The isolation of the Cd-peptide complexes at neutral pH

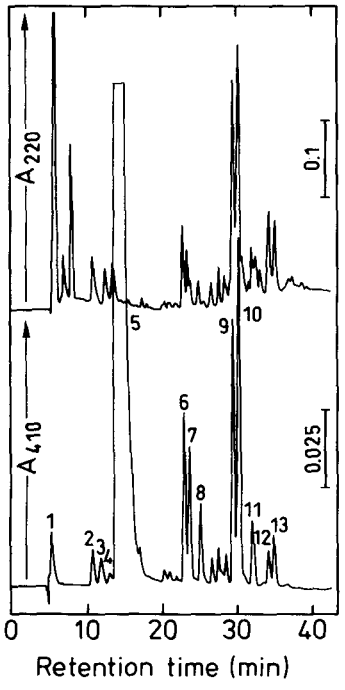

Figure 1. HPLC analysis of a crude extract of rice roots with detection by postcolumn derivatization with DTNB (A_{410}) and of the purified γEC peptides with detection by UV (A_{220}). The crude extract was prepared with roots of 9-d-old rice seedlings exposed to 100 μM Cd for 3 d, and the γEC peptides were purified by QAE ion-exchange chromatography. Peaks identified by reference substances are: 1, Cys; 2, hm-GSH; 3, GSH; 4, γ-Glu-Cys; 5, DTE. Peaks identified by amino acid analysis or relative retention time are: 6, hm-PC_2; 7, PC_2; 8, desGly-PC_2; 9, hm-PC_3; 10, PC_3; 11, desGly-PC_3; 12, hm-PC_4; 13, PC_4.

using published methods (Rauser, 1990) was prevented by the high oxidizing potential of the root extract. Even in the HCl extracts the γEC peptides are mostly in the oxidized form: reduction with DTE increases the γEC peptide content by a factor of 1.2 to 5, depending on the chain length and isoform of γEC peptide (results not shown). After the γEC peptides were purified and concentrated by anion-exchange chromatography, the major UV-absorbing peaks of an HPLC analysis of the column eluant coincide with thiol peaks of the crude root extract (Fig. 1), thus enabling the isolation of individual peptides by fractionation of the HPLC eluant.

Six individual peaks (Nos. 6–11 in Fig. 1) were subjected to total hydrolysis and quantitative amino acid analysis. In these fractions 93% of the amino acid residues present could be accounted for by Glu, Cys, Gly, and Ser. The ratios obtained (Table I) suggest that peaks 6 and 9 represent the peptides $(\gamma EC)_2S$ and $(\gamma EC)_3S$, respectively, peaks 7 and 10 are identical with PC_2 and PC_3, respectively, and peaks 8 and 11 represent the desGly-PCs $(\gamma EC)_2$ and $(\gamma EC)_3$, respectively.

The structure of the purified isopeptides was verified by enzymic hydrolysis of the C-terminal amino acids with carboxypeptidase A. Incubation of $(\gamma EC)_2S$ and $(\gamma EC)_3S$ resulted in the release of Ser only, and with PC_2 and PC_3 only Gly was split off. With all four isopeptides the amount of peptide hydrolyzed corresponded to the amount of $(\gamma EC)_2$ and $(\gamma EC)_3$, respectively, released.

Growth of rice plants in 1 mM buthionine sulfoximine, a potent inhibitor of γ-glutamylcysteine synthetase, reduced the GSH and hm-GSH content of the roots to 10% of the control plants; exposure of these plants to 100 μM Cd showed that induction of peptides of the $(\gamma EC)_nS$, PC, or desGly-PC type was completely abolished. Therefore, the synthesis of all of these peptides depends on the presence of a γ-glutamylcysteine-containing precursor.

The direct sequence and the establishment of the γ-glutamyl linkages of the suspected $(\gamma EC)_3S$ peptide isolated by the procedure given above was provided by tandem MS. This method has previously been applied for structure analysis of PC_2 and/or PC_3 (Steffens et al., 1986; Isobe et al., 1990; Kneer et al., 1992). The positive fast atom bombardment MS yielded an $(M+H)^+$ ion of m/z 802, corresponding to M_r 801. This ion was selected for collision with argon atoms to produce the fragment pattern shown in Figure 2. The peaks for the N-terminal ions B_1, B_2, and B_4 and for the C-terminal ions Y''_2, Y''_3, Y''_5, and Y''_6, following the nomenclature of Roepstorff and Fohlman (1984), are clearly seen. According to Isobe et al. (1990), the absence of B_5 and the relative abundance of Y''_6 is a clear indication that Glu is bound to Cys via the C-5 atom (γ-linkage).

From these experiments we conclude that in rice Cd-inducible peptides of the general structure $(\gamma EC)_nS$ are found with a chain length pattern identical with the one observed in the PC and h-PC series. These peptides will be named hm-PCs. Larger γEC peptides that have not been analyzed by amino acid composition were also found in the rice root extracts and were identified as hm-PC_4 and PC_4 by their retention times during HPLC according to the formula given

Table I. *Amino acid composition of rice peptides purified by QAE ion-exchange chromatography and by HPLC*

HPLC fractions corresponding to peaks 6 to 11 of Figure 1 were oxidized with performic acid, hydrolyzed, and subjected to quantitative amino acid determination. The values given have been normalized to Glu content, which was set 2 for peak Nos. 6 to 8 and 3 for peak Nos. 9 to 11.

	Peak No.					
	6	7	8	9	10	11
Ala	0.18	0.10	0.08	0.15	0.07	0.10
Arg	0.01	0.05	0	0.06	0	0.04
Asp	0.14	0.10	0.09	0.13	0.03	0.17
Cys[a]	**1.81**[b]	**1.86**	**1.83**	**2.83**	**2.91**	**2.65**
Glu	**2**	**2**	**2**	**3**	**3**	**3**
Gly	0.23	**1.01**	0.33	0.21	**0.94**	0.20
His	0.01	0	0	0.02	0	0
Leu	0.09	0.07	0.05	0.09	0.02	0.05
Lys	0.01	0.04	0.03	0.09	0	0.04
Ser	**0.97**	0.22	0.21	**0.97**	0.10	0.15
Thr	0.08	0.09	0.05	0.09	0.02	0.06
Tyr	0.03	0	0	0	0	0
Val	0.09	0.07	0.07	0.10	0.04	0.09

[a] Cys was measured as cysteic acid. [b] Values shown in boldface type are the constituent amino acids of the γEC peptides.

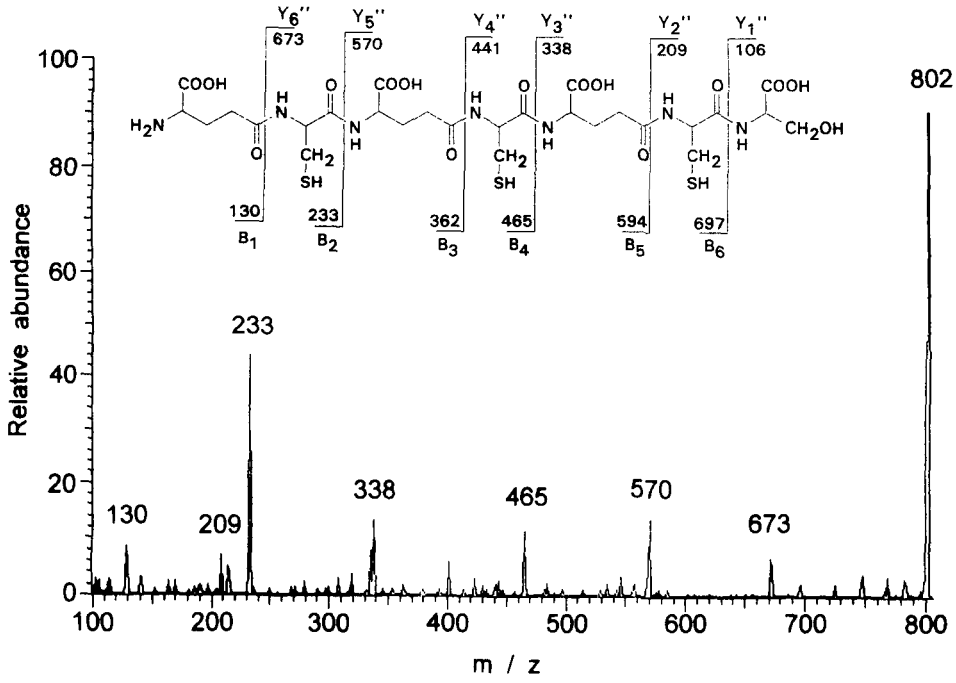

Figure 2. Fast atom bombardment tandem MS spectrum of (γ-Glu-Cys)$_3$-Ser showing the sequence with fragment ions containing the carboxyl (Y''_n) and amino termini (B_n).

by Gekeler et al. (1989), which in our experience also fits for $(\gamma EC)_nS$ and $(\gamma EC)_n$ peptides using the retention times of hm-GSH and γ-Glu-Cys as a basis.

hm-PC Synthesis in Different Plants

The identification of hm-PCs in rice implicates the general occurrence of these γEC peptide isoforms among those species of the family Poaceae, which do have hm-GSH (Klapheck et al., 1992). Several species of some of the most important food plants and some grasses including wheat, rye, oat, and maize were cultivated for 3 d at 250 μM Cd, and the crude HCl extracts from the roots were analyzed. In all species but maize hm-PC_2 and hm-PC_3 are present, although in lower amounts than found in rice. DesGly-PCs are also found in all species, and in *P. violaceum* and *P. setaceum* this isoform is even the predominant γEC peptide.

Kinetics of γEC Peptide Induction and Changes in the GSH/hm-GSH Content during Cd Exposure

Exposure of 6-d-old rice plants to 100 μM Cd is inhibitory to normal plant growth and leads to a reduced fresh weight increase of the roots (increase within 4 d was 193% for the Cd-exposed plants and 248% for the control plants) and of the shoots (280 and 326%, respectively). Although the roots of these plants already have a low γEC peptide content before Cd exposure, which is probably due to the presence of Cu and Zn in the growth solution, the content of all three types of γEC peptides increases immediately after the addition of Cd (Fig. 3). During the first 12 h after Cd administration, hm-PC_2 is the predominant γEC peptide of the roots. PC_2 synthesis is delayed, but it increases during the following 72 h. After 12 h the content of both peptides is exceeded by the γEC content present in the $n = 3$ isoforms of $(\gamma EC)_nG$ and $(\gamma EC)_nS$, with an increasing ratio of PC_3:hm-PC_3 during prolonged Cd exposure. PC_4 and hm-PC_4 reach only low amounts compared to the other thiols, but here, also, the relative content of the Gly isoform is increasing during incubation. The desGly-PCs are already present before Cd addition, and their content increases continuously. In the average of three kinetics experiments, the relative contribution of the $(\gamma EC)_n$ peptides to all peptides with n γEC units increases from 15 to 25% for $n = 2$ and from 7 to 15% for $n = 3$ during the last 72 h of incubation. In the control plants, which were cultivated under the same conditions but without Cd, the initial low content of γEC peptides in the roots, which predominantly is based on peptides with $n = 2$ γEC units, is reduced by half during the 4-d growth period.

To evaluate the changes in the GSH and hm-GSH concentrations due to Cd exposure, the changes due to plant development have to be considered. In the roots of 6-d-old rice plants, the GSH content is 1.7-fold higher than the hm-GSH content. During the time course of the kinetics experiment both thiols increase by half in the control plants (Fig. 3). Addition of Cd to the plants leads to an immediate and significant decrease of the GSH content: after 6 h of exposure the GSH content is reduced from 172 to only 73 nmol g^{-1} fresh weight, and it is later restored only insignificantly. The hm-GSH content, however, is doubled within the first 24 h and then subsequently declines slowly.

In the shoots of the rice plants, γEC peptides are induced upon Cd exposure to a much smaller extent (Fig. 4). Peptides with $n = 2$ γEC units are nearly twice as abundant as peptides with $n = 3$ γEC units, and hm-PCs predominate by far. In

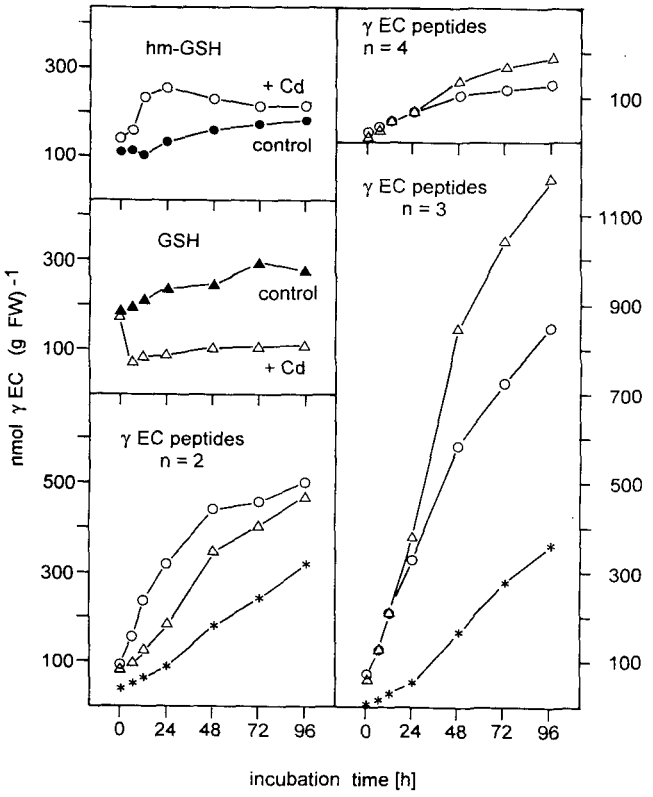

Figure 3. Thiol content of rice roots exposed to Cd for different times. Rice seedlings (6 d old) grown hydroponically were exposed to a growth solution containing 100 μM Cd. After the incubation times indicated, 40 seedlings were removed, and the roots were analyzed for GSH, hm-GSH, and γEC peptide content. The values given represent the means of three independent experiments. O, $(\gamma\text{-Glu-Cys})_n$-Ser peptides; Δ, $(\gamma\text{-Glu-Cys})_n$-Gly peptides; *, $(\gamma\text{-Glu-Cys})_n$ peptides of Cd-exposed plants; hm-GSH (O) and GSH (Δ) content of Cd-exposed plants, hm-GSH (●) and GSH (▲) content of control plants.

the control plants γEC peptide content is just above detection limits.

The shoots of 6-d-old plants have nearly equal concentrations of GSH and hm-GSH, and both thiols show a linear increase during the time course for the control plants. An influence of Cd on the thiol content is visible only after 6 to 12 h of incubation. Similar to the changes observed in the roots, Cd exposure leads to a significant decrease of the GSH content, which starts to recover only after 72 h, and to a much larger increase of the hm-GSH content compared with the control shoots.

Influence of Cd Concentration on γEC Peptide and GSH/hm-GSH Content

Rice plants cultivated for 7 d in Hoagland solution were exposed to Cd at different concentrations for 48 h. Judged by the fresh weight of shoots and roots, growth inhibition is visible at Cd concentrations exceeding 25 μM (Table II). Although the highest contents of γEC peptides are found at 500 μM Cd, there seems to be an increasing limitation of γEC peptide synthesis already at Cd concentrations exceeding 10 μM (Table II). In the roots hm-PC_2 and hm-PC_3 are the predominant γEC peptide isoforms at low Cd concentrations, but at higher concentrations the γEC content present in PC_3 increases steadily and exceeds all other isoforms. In the shoots even at high Cd concentrations the total γEC peptide content is based mainly on hm-PC_2 and hm-PC_3.

The Cd concentration also has a profound influence on the GSH and hm-GSH content of the rice plants (Table II). In the roots the GSH content is drastically decreased with increasing Cd concentration; however, the hm-GSH content increases to a small extent. In the shoots, the decrease in GSH content is smaller than in the roots, whereas the hm-GSH content is nearly doubled compared with the controls. hm-PCs and PCs can also be induced by incubation of rice plants with Zn or Pb at 500 μM but with a much smaller effect than with Cd (results not shown).

DISCUSSION

The data presented clearly show that in plants of the family Poaceae a series of γEC peptides with two to four γEC units and a C-terminal Ser is present, which is homologous to the h-PC series found in legumes (Grill et al., 1986) and the PC series found in most other plants (Gekeler et al., 1988, 1989; Rauser, 1990; Steffens, 1990). In analogy to the tripeptide hm-GSH these Ser-containing peptides are termed hm-PCs.

There is good evidence that hm-PCs are used for the assembly of Cd-peptide complexes. Rauser et al. (1986, 1988)

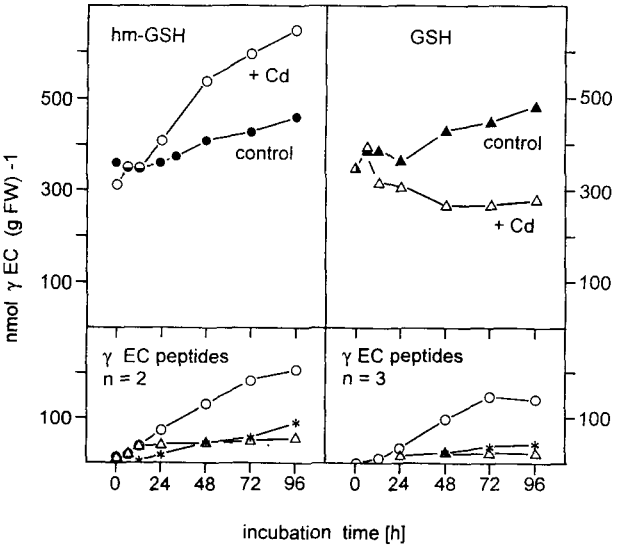

Figure 4. Thiol content of rice shoots exposed to Cd for different times. Rice seedlings (6 d old) grown hydroponically were exposed to a growth solution containing 100 μM Cd. After the incubation times indicated, 40 seedlings were removed, and the shoots were analyzed for GSH, hm-GSH, and γEC peptide content. The values given represent the means of three independent experiments. O, $(\gamma\text{-Glu-Cys})_n$-Ser peptides; Δ, $(\gamma\text{-Glu-Cys})_n$-Gly peptides; *, $(\gamma\text{-Glu-Cys})_n$ peptides of Cd-exposed plants; hm-GSH (O) and GSH (Δ) content of Cd-exposed plants, hm-GSH (●) and GSH (▲) content of control plants.

Table II. *Content of total GSH and hm-GSH and of γEC peptides in roots and shoots of rice seedlings exposed to various concentrations of Cd*

Roots of 7-d-old rice seedlings (60 plants each) were exposed to 350 mL of metal-containing nutrient solution for 48 h. GSH and hm-GSH contents of the HCl extracts were analyzed after reduction and derivatization (Klapheck, 1988). γEC peptides were analyzed by the postcolumn DTNB derivatization method. The values given are the means of three independent experiments.

Cd	Thiol Content										Fresh Wt (60 plants)
	hm-GSH	GSH	hm-PC_2	PC_2	desGly-PC_2	hm-PC_3	PC_3	desGly-PC_3	hm-PC_4	PC_4	
μM	*nmol of γEC g^{-1} fresh wt*										g
Shoot											
0	315	285	11	10	3						1.46
10	340	231	64	19	11	41	12	4	7	3	1.43
25	476	231	93	31	25	48	11	2	6	3	1.45
100	595	185	127	40	32	95	16	13	20	3	1.35
250	662	176	200	52	55	174	26	18	25	6	1.30
500	715	165	218	55	62	208	18	19	30	9	1.16
Root											
0	168	321	47	38	16	13	7				0.65
10	213	102	207	69	50	170	68	10	18	7	0.66
25	181	87	213	114	69	224	170	21	28	20	0.62
100	198	67	303	180	105	360	317	46	50	37	0.55
250	202	61	334	199	134	398	487	87	62	63	0.51
500	239	58	338	209	132	391	521	96	65	80	0.42

showed that some of the polypeptides that were set free from Cd-binding complexes purified from the roots of *A. gigantea* have Ser instead of Gly in addition to equimolar quantities of Glu and Cys, whereas the amino acid composition of other peptides corresponds to the structure of PCs. The HPLC elution pattern of these peptides reported by Rauser et al. (1988) clearly shows the same successive double peaks that we found in our HPLC experiments, which indicates that the Cd-binding complexes of *A. gigantea* consist of hm-PCs and PCs with two to four γEC units. The C-terminal amino acid seems not to be critical to Cd-peptide complex formation. h-PCs that have a C-terminal β-Ala and the desGly-PCs also are found in metal-binding complexes (Grill et al., 1986; Bernhard and Kägi, 1987; Mehra and Winge, 1988). In fact, the maximal Cu(I)-binding stoichiometry is higher for desGly-PC_3 than for PC_3 (Mehra and Winge, 1988). The presence of Ser might even improve the metal-binding capacity because of the negatively charged hydroxyl group. Analysis of the amino acid sequence of 30 metallothioneins from mammals shows that in addition to the high Cys content (mean value 33 mol%) Ser is the second predominant amino acid (12–18 mol%) with most Ser being adjacent to a Cys residue (Kägi and Kojima, 1987). It is tempting to speculate that the high content of Ser in metallothioneins and the presence of Ser in the hm-PCs have a positive effect on the metal-binding properties of these peptides.

The γEC peptide composition of the rice roots changes significantly and in a similar way during the time course after Cd addition as well as with the increase of Cd concentration. During the first 24 h of incubation and at low Cd concentrations hm-PC_2 is the predominant peptide. Prolonged incubation time or higher concentrations not only lead to γEC peptides of higher chain length but also favor the synthesis of the PC isoforms: the ratio of PC_3 to hm-PC_3 and of PC_4 to hm-PC_4 increases steadily. The successive appearance of the γEC peptides of higher chain length is explained by the presumption that in vivo the smaller peptides are used as precursors for the synthesis of the larger peptides, as has been shown for the in vitro synthesis of PCs by PC synthase (Grill et al., 1989; Loeffler et al., 1989). A similar time course of accumulation of γEC peptides varying in chain length has been found for *Schizosaccharomyces pombe* (Hayashi and Nakagawa, 1988) and for *Rauwolfia serpentina* cell cultures (Grill et al., 1985). In maize roots, preference of PCs with higher chain length has been shown to be the result of both prolonged incubation time and increasing Cd concentrations (Tukendorf and Rauser, 1990). The predominant synthesis of hm-PC_2 during the first hours and the preferred synthesis of the PC isoforms with regard to peptides of $n = 3$ to 4 γEC units, observed after 2 d of incubation at 100 μM Cd or at higher Cd concentrations, may be an indication of the synthesis pathway. If we assume that hm-PCs are synthesized in the same way that PCs are (Grill et al., 1989; Loeffler et al., 1989), namely by a transpeptidation reaction catalyzed by a synthase and using hm-GSH and hm-PC_n as substrates for the synthesis of hm-PC_{n+1}, this result may reflect the substrate specificity of the synthase: hm-GSH may be a good substrate for the synthesis of the hm-PC_2 peptide, whereas the hm-PCs may be a poor substrate for elongation of the peptide chain. An alternative pathway for the synthesis of hm-PCs is the addition of Ser to the desGly-PCs as proposed for the Gly addition by GSH synthetase in PC synthesis in *S. pombe* (Hayashi et al., 1991). However, we have not been able to demonstrate an hm-GSH synthetase that adds Ser to γ-Glu-Cys, and the pathway of hm-GSH synthesis has still to be elucidated.

The rice shoots produce γEC peptides more slowly and in much smaller concentration than do the roots. Probably the roots control the passage of Cd to the shoots, and therefore the Cd concentration reaching the shoot is much smaller. In maize plants grown in 3 μM Cd for 4 d, the concentration of Cd on a fresh weight basis was 7-fold higher in roots than

in shoots (Meuwly and Rauser, 1992). In the rice shoots hm-PCs with two or three γEC units are the predominant form of γEC peptide. The γEC peptide composition in the shoots has similarities to the composition in the roots during the first 12 h of Cd incubation or at low Cd concentrations and, therefore, may reflect not a different γEC peptide pattern of shoots in principle but the reaction of rice tissues to Cd exposure at lower concentrations.

In addition to the PCs and hm-PCs, desGly-PCs are found in appreciable amounts in rice roots and shoots and in roots of other graminaceous plants. The occurrence of this isoform in plants was first shown in maize (Bernhard and Kägi, 1987), and recently Rauser and Meuwly (1993) reported that desGly-PC_2 is the most abundant γEC peptide in maize roots. DesGly-PCs also occur in the fungi *S. pombe* (Mehra and Winge, 1988) and *Candida glabrata* (Barbas et al., 1992). Apparently this γEC peptide isoform is more abundant among different taxa than stated by Gekeler et al. (1989). The relative amount of the desGly-PCs in rice increases slowly in the time course, therefore supporting the hypothesis that this isoform arises catabolically from the Gly- or Ser-terminal γEC peptides by action of carboxypeptidase. The presence of these peptides at the beginning of the Cd incubation and at low Cd concentrations, however, may be an indication that de novo desGly-PC synthesis is possible, probably by polymerization of γEC and GSH, as has been shown in vitro using cell-free extracts of the fission yeast (Hayashi et al., 1991).

The analysis of the GSH and hm-GSH content of the roots and shoots shows that the content of both thiols is influenced in a quite different way by Cd exposure. The GSH content declines very rapidly in the roots and more slowly in the shoots after Cd addition, more drastically depending on the Cd concentrations. In contrast, the hm-GSH content increases after Cd exposure in the roots as well as in the shoots, and the increase of hm-GSH is proportional to the Cd concentration in the shoots. A Cd-induced decline in the pool of GSH has been observed for many plants and fungi (Grill et al., 1987; Tukendorf and Rauser, 1990; Meuwly and Rauser, 1992; Bergmann and Rennenberg, 1993) and reflects GSH consumption by the starting PC synthesis. During early Cd exposure, the decline in GSH matches the PC biosynthesis in cell cultures of *R. serpentina* (Grill et al., 1987) and in maize roots (Tukendorf and Rauser, 1990). The GSH decline observed in the rice roots using the growth-limiting Cd concentration of 100 μM is much lower than the γEC peptide increase; therefore, additional GSH synthesis has to take place soon after Cd addition.

The increase in hm-GSH content, however, in spite of the hm-PC synthesis that is most prominent during the early time of Cd exposure, is surprising. The only report about a Cd-induced increase of GSH concerns *C. glabrata* cells treated with $CdCl_2$ (Barbas et al., 1992). The 3- to 6-fold increase in the GSH content of this fungus is attributed to the formation of a Cd:sulfide crystallite, with GSH as the major coating peptide. The increasing hm-GSH content in rice roots and shoots, therefore, may confirm the participation of hm-GSH in Cd-complex formation, but it may also be the result of a considerable increase of the hm-GSH synthesis rate.

In conclusion, the first defense of rice plants to Cd exposure is the rapid synthesis of hm-GSH and hm-PC_2. Later, hm-PCs of higher chain length and PCs are formed, with increasing predominance of the PCs after prolonged incubation time or with higher Cd concentrations. Further experiments have to prove that hm-PCs are constituents of the Cd-binding complexes and have to reveal the mode of their biosynthesis.

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