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Cadmium-Sulfide Crystallites in Cd-$(\gamma EC)_nG$ Peptide Complexes from Tomato[1]

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ABSTRACT

Hydroponically grown tomato plants (*Lycopersicon esculentum* P. Mill. cv Golden Boy) exposed to 100 micromolar cadmium sulfate produced metal-$(\gamma EC)_nG$ peptide complexes containing acid-labile sulfur. The properties of the complexes resemble those of the cadmium-$(\gamma EC)_nG$ peptide complexes from *Schizosaccharomyces pombe* and *Candida glabrata* known to contain a cadmium sulfide crystallite core. The crystallite is stabilized by a sheath of peptides of general structure $(\gamma Glu\text{-}Cys)_n$-Gly. The cadmium-peptide complexes of tomato contained predominantly peptides of n_3, n_4, and n_5. Spectroscopic analyses indicated that the tomato cadmium-sulfide-peptide complex contained CdS crystallite core particles smaller than 2.0 nanometers in diameter.

Heavy metals have been shown to induce the formation of cysteine-rich peptides having the general structure $(\gamma Glu\text{-}Cys)_n$-Gly ($n$ = 2–8) in vascular and nonvascular plants, *Candida, Schizosaccharomyces,* and *Euglena* (5, 7–9, 11, 19, 25, 28). These peptides, designated by various terms including cadystin and phytochelatin, are presumed to function in metal detoxification by sequestering metal ions within the protoplasm (for reviews see refs. 17 and 24). Synthesis of the peptides from glutathione by a constitutive, metal-activated enzyme, termed phytochelatin synthase, has been documented (6). An additional component of the complex is acid-labile sulfur (9, 13, 18, 25). Cadmium has been shown to increase sulfate reduction in plants (16). Incorporation of acid-labile sulfur into Cd-peptide complexes increases the metal binding affinity and cadmium stoichiometry of the peptides (20). Native isolates of Cd-$(\gamma EC)_nG$ peptide complexes from *Candida glabrata* contain a S:Cd ratio between 0.3 and 0.7 (2). Complexes having a S:Cd ratio of 0.7 contain a 2.0 nm diameter CdS crystallite coated with nearly 30 $(\gamma EC)_nG$ peptides of predominantly n_2 and the desGly form (2). The size of the crystallite determines the optical properties of the complex, as is known for synthetic semiconductor CdS clusters (1, 15, 22). The separation of the lowest excited electronic state from the ground state in 2.0 nm lattice particles is of an energy corresponding to 315 nm (2). Acidification, which volatilizes the sulfur as H_2S, abolishes this transition. Complexes with lower S:Cd ratios have electronic transitions that are blue-shifted to higher energy, indicating that the edge absorption transitions can be used as a measure of crystallite size (2).

The importance of sulfide ions in metal ion detoxification is substantiated by cadmium-hypersensitive *Schizosaccharomyces pombe* mutants apparently deficient in the metal-mediated sulfide response (14). In addition, cadmium-tolerant *Silene vulgaris* plants exhibit a higher S:Cd ratio in the $(\gamma EC)_nG$ peptide complexes than cadmium-sensitive plants (26). Sulfide ions have also been reported to be associated with peptide complexes isolated from *Lycopersicon* and *Euglena,* but only partial characterization of these metal complexes has been reported (25, 28). In this report, we present data establishing the presence of sulfide in Cd-peptide complexes from roots and leaves of tomato plants and show that electronic transitions typical of CdS semiconductor crystallites exist in these complexes. Formation of CdS colloidal particles may be a general mechanism by which $(\gamma EC)_nG$ peptides act to sequester the metal.

MATERIALS AND METHODS

Tomato Plant Cultures

Seeds of tomato (*Lycopersicon esculentum* P. Mill. cv Golden Boy) were germinated in vermiculite and allowed to grow until plants were 15 cm tall. Individual plants were separated, and the roots were washed to remove residual vermiculite. Plants were grown hydroponically in a modified Hoagland solution (10) containing Fe-EDTA, and cadmium was added after 1 week. Cadmium levels were monitored by flame atomic absorption spectrophotometry on a Perkin-Elmer 305 spectrometer and maintained at 25, 50, or 100 μM by addition of $CdSO_4$. Nutrient solutions were changed biweekly. Shoots and roots were collected from plants harvested 4 weeks after the addition of cadmium sulfate. The tissue was frozen immediately at −70°C until it was processed.

Peptide Preparation

Tomato shoot or root tissues were homogenized in equal parts (w/v) of 50 mM Tris/HCl, 0.2% 2-mercaptoethanol, 2% (w/v) polyvinyl polypyrrolidone, pH 7.6, with a Virtis tissue homogenizer, filtered through cheesecloth, and centrifuged at 5000g for 10 min. The supernatant was further clarified by

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centrifugation at 20,000*g* for 15 min prior to chromatography on an anion-exchange resin (Whatman DE-52 in a 2.5 × 10 cm column). The column was eluted with a 400 mL gradient of 0 to 0.5 M KCl in 50 mM Tris/HCl, pH 7.6. Osmolarity of the buffer was monitored by freezing point depression with an Osmette A osmometer. The eluate was monitored by flame ionization atomic absorption spectrophotometry, and fractions containing Cd(II) were pooled and concentrated by lyophilization. The samples were then applied on a Sephadex G-50 column (2.5 × 100 cm) equilibrated and eluted with 50 mM Tris/HCl, 0.1 M KCl, pH 7.6. In some experiments, fractions from the G-50 eluate were pooled, lyophilized, and rechromatographed on a Sephadex G-50 column equilibrated with 50 mM Tris/HCl lacking KCl to improve separation of the various Cd-peptide complexes (20). Amino acid analysis and peptide separation by C_{18} reverse-phase HPLC were conducted as previously described (20). Sulfide concentrations were quantified by the synthesis of methylene blue as described by King and Morris (12). UV spectroscopy was conducted on a Beckman DU-64 spectrophotometer.

RESULTS

Tomato plants exposed to 100 μM Cd(II) for 4 weeks showed signs of toxicity, including a slight chlorosis and occasional discoloration of the leaves near veins. This concentration of cadmium was not lethal to this cultivar, however, as plants matured and produced fruit even at metal concentrations of 200 μM in the nutrient medium. Control plants and those growing in cadmium concentrations of <100 μM were healthy and grew vigorously throughout the experiment.

The Cd-peptide extracts shown in Figure 1 were prepared from roots of metal-treated tomato plants and eluted as a broad pair of overlapping peaks from the anion-exchange resin with the first peak eluting at an ionic strength of approximately 550 mosmol and the second near 650 mosmol (Fig. 1a). Chromatographic separation of the individual pooled fractions (Fig. 1a, tubes 61–70 and 48–58) on Sephadex G-50 revealed two overlapping peaks containing Cd(II) eluting with distribution coefficients (K_{av}) of approximately 0.62 and 0.70, peaks 1 and 2, respectively (Fig. 1b, peak 1). The profile of the sulfide concentration was not coincident with the cadmium concentration. Rather, the peak sulfide-containing fractions were displaced to fractions with greater Stokes radii. Rechromatography of cadmium- and sulfide-containing fractions from the leading edge of peak 1 (Fig. 1b, $K_{av} \geq 0.45$ and ≤ 0.60) on Sephadex G-50 under conditions of low salt resulted in a single peak with a K_{av} of 0.33 for both cadmium and sulfide.

Amino acid analyses of materials from both peaks 1 and 2 revealed only Glx, Cys, and Gly, which is indicative of $(\gamma EC)_nG$ peptides. Reverse-phase HPLC of materials from both of the Sephadex G-50-prepared peaks revealed a series of $A_{214\,nm}$ absorption peaks with retention times identical to those of standard $(\gamma EC)_nG$ peptides of n_3, n_4, n_5, and n_6 (Fig. 2a and b). The n_4 peptide was clearly the predominant species in the peak 1 material, with significant amounts of the larger peptides being present. A small amount of n_3 was also observed in this material. In comparison, the peak 2 material contained almost as much n_3 as n_4 peptide in the complex,

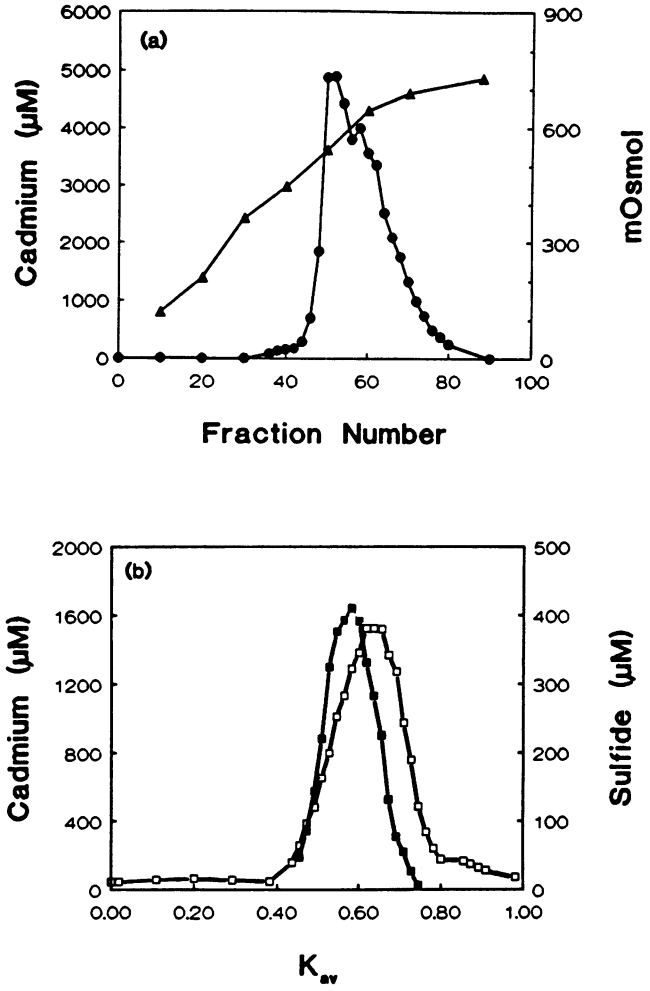

Figure 1. a, Elution profile of tomato root Cd-peptide complex from Whatman DE-52 column, 4 mL/tube; (—●—) cadmium, (—▲—) ionic strength. b, Elution profile of tomato Cd-peptide complex from Sephadex G-50 column (tubes 61–70, Fig. 1a), flow rate 0.7 mL/min, buffer: 50 mM Tris, 0.1 M KCl, pH 7.6; (—□—) cadmium, (—■—) sulfide.

with only limited amounts of the n_5 and n_6 peptides. Profiles of peptide complexes from leaves of plants grown in 100 μM Cd and both roots and leaves of tomatoes grown at lower metal concentrations were similar to those of peak 2, with n_3 and n_4 being the predominant peptide species.

Sulfide measurements over the profiles of peaks 1 and 2 were qualitatively similar because of the large amount of overlap of the pooled materials from the anion-exchange column, with the S:Cd ratios being highest at the leading edge of the elution profile (Fig. 1b). The absolute S:Cd ratios differed considerably between peaks 1 and 2, however, with peak 1 having a maximum S:Cd ratio of 0.41 (Table I) and peak 2 having a maximum S:Cd ratio of 0.13. The rechromatographed peak 1 fraction yielded a single Cd-peptide complex with a mean S:Cd ratio of 0.35.

Peptide extracts from leaves of 100 μM Cd-treated plants and tissues of tomato plants grown with lower metal concentrations in the nutrient medium were similar to complexes

isolated from roots. However, they generally contained lower total peptide, cadmium, and sulfide concentrations and had S:Cd ratios that were less than 0.4.

UV absorption spectrophotometry was performed on eluate fractions from peak 1, peak 2, and the rechromatographed peak 1 materials. Figure 3a shows the profiles of three fractions taken from peak 1. The fraction with a K_{av} of 0.53, on the high Stokes radius edge of the profile, showed two transitions near 260 and 285 nm. Later fractions from the elution profile (K_{av} 0.58) did not contain the 285 nm transition but retained a prominent transition centered near 260 nm, which was absent in elution fractions $K_{av} \geq 0.65$. Quantitation of sulfide in the fractions clearly demonstrated correlations between S:Cd ratios and the UV transitions near 285 nm (Table I).

The peptide complex ($K_{av} = 0.53$) was acidified to pH 1.5 to volatilize the sulfide. Absorption spectroscopy of the sample after reneutralization showed that the transitions at 260 and 285 nm did not reappear (Fig. 3b), but that there was the appearance of a transition near 250 nm. Loss of the two transitions as a result of acidification is directly correlated with loss of sulfide, which was made evident both by the characteristic odor of H_2S given off during the treatment and by the loss of reactivity of the reneutralized complexes in the direct sulfide assay. Addition of 1 mol equivalent of sulfide (based on Cd) to the Cd-peptide complex *in vitro* resulted in a red shift of the transition to near 315 nm (Fig. 3b). Peak 2 fractions contained negligible amounts of sulfide and did not exhibit any red-shifted electronic transitions.

Table I. *Sulfide/Cadmium Ratios of Selected Tubes from Sephadex G-50 Elution Profiles, Peak 1 (Fig. 1b) and Peak 2 (not shown) of Tomato Root Peptide Complexes*

Peak	K_{av}	S/Cd
1	0.53	0.41
	0.58	0.32
	0.66	0.15
2	0.58	0.13
	0.63	0.09
	0.71	0.04

DISCUSSION

Tomato plants respond to metal stress by synthesis of $(\gamma EC)_nG$ peptides that chelate intracellular metal ions. The peptides are identical to those described from other plant species and appear to be a common response to heavy metal stress in higher plants (17, 24). Properties characteristic of the $(\gamma EC)_nG$ peptides include: (a) only Glu, Cys, and Gly amino acids being present (except in those species producing homoglutathione); (b) peptide length heterogeneity of the complexes, with n_2 to n_8 being common; and (c) salt-dependent

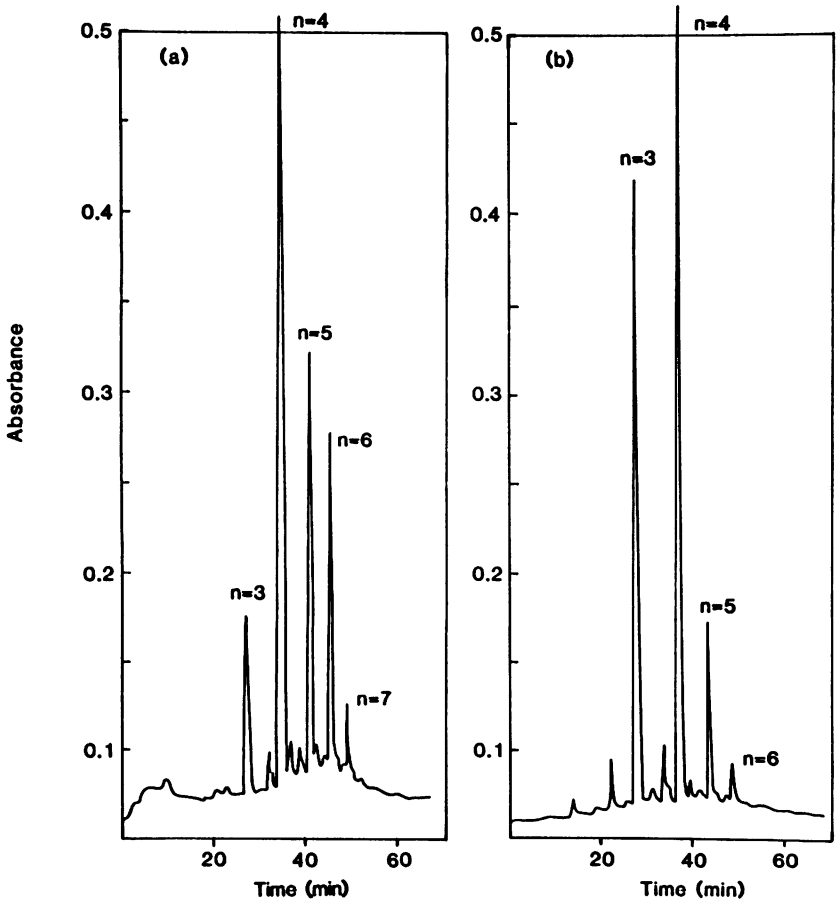

Figure 2. Profile of acidified tomato Cd-peptide complexes (apoproteins) from C-18 reverse-phase HPLC column eluted with a 0 to 60% acetonitrile gradient. a, High sulfide-containing complex (Fig. 1b, K_{av} 0.62); b, low sulfide complex (K_{av} 0.70, after Sephadex G-50 separation of materials in tubes 48–58, Fig. 1a).

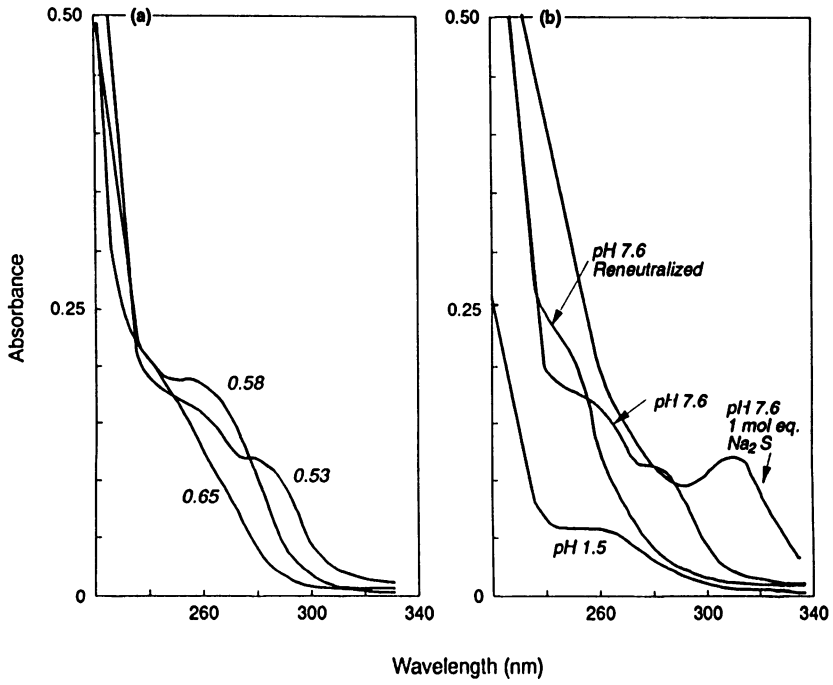

Figure 3. a, Absorption spectra of tomato Cd-peptide complexes from selected tubes (Fig. 1b, K_{av} = 0.53, 0.58, 0.65) of a Sephadex G-50 profile eluted with 50 mM Tris 0.1 M KCl, pH 7.6; samples contained 4 μg Cd/mL. b, Absorption spectra of tomato Cd-peptide complex before acidification (K_{av} = 0.53), acidified to pH 1.5 with HCl, reneutralized with NaOH to pH 7.6, and reneutralized plus 1 mol equivalent of Na_2S.

variation in apparent M_r of the complexes, as determined by gel filtration, presumably due to electrostatic effects.

The occurrence of two cadmium-peptide complexes having similar peptide composition but differing in apparent M_r after separation by gel filtration is consistent with our previous findings for metal-peptide complexes isolated from fission yeast (18, 20). The difference between the two peaks is due to the labile sulfur content. Thus, two populations of metal-peptide complexes differing in the content of bound sulfide ions can form in plants as well as yeast. The high sulfide form of the complex appears to be a response to high concentrations of cadmium and may be important in the overall tolerance to metal stress, as sulfide greatly increases the metal binding affinity and stoichiometry of the complex (20).

Selection of larger peptides during formation of high sulfide-containing Cd-peptide complexes has been shown experimentally (20) and is clearly demonstrated by the HPLC data (Fig. 2a and b). Average peptide length also showed a general increase with increasing cadmium levels in a given cultivar but varied with cultivar and culture conditions. Experiments similar to those described above were conducted with *L. esculentum* cv Stone and resulted in a complex in which n_3 was the predominant peptide length, even though the maximum S:Cd ratio was 0.52. The data for tomatoes are consistent with our earlier findings and suggest that the tomato CdS-$(\gamma EC)_nG$ peptide complexes are similar to those found in *S. pombe*.

Sulfide production in tomatoes and other organisms that form $(\gamma EC)_nG$ peptide complexes appears to be a response to exposure to high concentrations of cadmium (9, 21, 28). These data and earlier studies with *S. pombe* (18) demonstrate that exposure to higher concentrations of cadmium causes incorporation of sulfide *in vivo*. With low concentrations of metal, tomatoes form predominantly the low sulfide complex. Elevation of cadmium concentrations resulted in an increase in the percentage of high sulfide complex. This was seen in the plants grown in 25 and 50 μM Cd and in the distribution of complexes in roots and leaves from the same plants. We have found sulfide-containing complexes under all of the conditions described, but generally the S:Cd ratios are $\leq$0.1 in plants exposed to $\leq$50 μM Cd. High sulfide-containing complexes were more common in roots than leaves of all of the tomato plants thus far examined, in direct correlation to the cadmium concentrations in those tissues. Variation in the site of metal localization in plants has been shown by Wagner and Yeargan (27) to depend upon a genetic component, in addition to metal concentrations, as different cultivars of tobacco accumulated metals preferentially in leaves or roots. Therefore, localization and total sulfide production in a given plant may also vary depending upon the plant's genotype and the concentration of cadmium to which it is exposed.

Dameron *et al.* (2, 3) demonstrated that peptides varying in length from n_1 (glutathione) to n_4 can act as coatings to stabilize CdS crystallites. The number of γEC dipeptide repeats influences the stability of the complexes. Complexes formed with shorter peptides (n_1 and n_2) are more labile, and accretion of the crystallites to larger particles is more facile. Variation in the length of the peptides in CdS-$(\gamma EC)_nG$ complexes is observed in different yeast species (2), and this variation is influenced by components in the growth medium (2). Similar variations in peptide composition of isolated complexes have been observed between tomato cultivars—Golden Boy, Stone, and Rutgers (this report, 21, and our unpublished data), with Golden Boy consistently producing the longest peptides under the conditions described.

The near UV transitions between 285 and 260 nm for tomato Cd-complexes containing sulfide resemble transitions seen in CdS quantum clusters. CdS clusters smaller than 2.0 nm in diameter exhibit a variation in electronic edge transitions with the size of the crystallite (1, 15, 22, 23). The smaller

the clusters, the more blue-shifted is the electronic transition, with the wavelength of specific UV transitions being dependent upon the S:Cd ratio. The position of the transitions in the tomato CdS-peptide complexes suggests that the core cluster size is below 2.0 nm in diameter, as was shown for crystallites from *S. pombe* (2, 4). Both of these species produce complexes that are coated with peptides that are predominantly larger than n_2.

It is clear that these metal clusters are quite dynamic. Although the peptide coating stabilizes the clusters to flocculation, sulfide ions readily mediate an accretion of small clusters into larger particles that are red-shifted in optical properties (1, 20). Therefore, the size of the particles *in vivo* is dictated primarily by the magnitude of the metal-regulated sulfide response and the number of γEC dipeptide repeats in the isopeptides. Stated with reference to UV absorption, this means that the absolute value of the blue shift of the UV transition depends upon S:Cd ratios, peptide lengths, and the mean particle size in a given sample.

Further demonstration of the dynamic nature of the complexes is provided by the acidification/reneutralization experiments. The reneutralized peptide complex shows transitions around 250 nm consistent with Cd(II)-cysteinyl thiol charge-transfer transitions. This spectrum resembles the spectrum of Cd-$(\gamma EC)_nG$ formed in the absence of sulfide ions. The addition of equimolar sulfide attenuates the 250 nm transition as Cd-sulfide bonding becomes predominant. Concomitant with the diminution in the charge transfer transitions is the appearance of the band gap optical bands at wavelengths between 260 and 318 nm. The actual wavelength maximum is dependent on the lattice size. In the titration presented in Figure 3b, the transition is near 315 nm, which is consistent with a 2 nm crystallite lattice diameter (2). The $(\gamma EC)_{3-5}G$ peptides effectively arrest crystallite growth at this size (4).

CONCLUSIONS

We have shown that tomato plants are capable of producing cadmium-$(\gamma EC)_nG$ peptide complexes containing sulfide ions. Characterization of the tomato Cd-complexes demonstrated that they are analogous to the metal:sulfide crystallite particles isolated from *Candida* and *Schizosaccharomyces* in amino acid composition, sulfide content, and spectral properties. Therefore, we conclude that tomatoes produce CdS crystallite particles coated with $(\gamma EC)_nG$ peptides in response to cadmium and, furthermore, that this may be a general response to exposure to high concentrations of cadmium in plants.

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