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Analysis of Cadmium-peptide Complexes in Sunflower (*Helianthus annuus* L.) Roots as Detected by Gel-filtration Chromatography*

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Abstract: Fifteen-old sunflower (*Helianthus annuus* L.) plants on hydroponic culture were treated with 75 μM Cd. After 3 days of Cd exposure, a decrease in growth parameters (root and stem length and leaf area) was observed. In extracts of Cd-treated roots, gel-filtration chromatography detected one Cd-binding peptide designated PC-Cd complex, with an apparent M_r of 17.8 kDa. In response to Cd, the roots exhibited a higher accumulation of non protein thiols (NPTs) compared with control plants. Moreover, phytochelatin (PC) levels, estimated from the difference between total NPTs and glutathione (GSH), increased significantly, raising the possibility that PCs play a significant role in heavy metal detoxification. The higher PC concentrations were accompanied by lower GSH concentrations.

Key words: Cadmium, gel-filtration chromatography, glutathione, nonprotein thiols, PC-Cd complex, phytochelatins, sunflower (*Helianthus annuus* L.)

Introduction

The soil contamination by heavy metals becomes a serious problem in many countries in the word (Keltjens and Van Beusichem, 1998). Cadmium is a widespread pollutant spilled in the environment as a consequence of agricultural, manufacturing, mining and waste disposal practices or other anthropic factors (Sanità di Toppi *et al*., 1999). The pesticides, fungicides and phosphate fertilizers may be other sources of Cd pollution (Song *et al*., 2004). In soil solution, Cd is present as precipitated forms, bound to organic matter or as soluble forms (Sposito *et al*., 1982b). Nevertheless, an acid pH of the soil solution enhanced the Cd solubility (Wagner, 1993). The ionic Cd is the most available to the plants, but at extremely low concentrations (Hirch and Banin, 1990). Cd exposure may lead to growth inhibition, root damage, chlorosis and it may affect transpiration (Das *et al*., 1998; Haag-Kerwer *et al*., 1999). It has been proposed that Cd can cause an oxidative stress by displacement of essential heavy metal ions in reaction centers of proteins, resulting in the loss of their biological function and the release of free ions (Hall, 2002). The plants respond to the heavy metal stress by different ways: exclusion, chelation or compartmentalization and expression of responses mechanisms such as the production of ethylene and stress proteins (Cobbett, 2000). One general mechanism for heavy metal detoxification in plants and other organisms is the chelation of the metal by a ligand and subsequent compartmentalization of the ligand-metal complex. A number of metal-binding ligands have now been recognized in plants. The roles of several ligands have been reviewed by Rauser (1999). The citrate and malate play important role as intracellular or extracellular agents for heavy metals chelation and sequestration. In a similar way, phytochelatins (PCs) which consist of repeating units of

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**Originally Published in Journal of Plant Sciences, 2006* γ-glutamyl-cysteine followed by a C-terminal Gly have been involved in heavy metal detoxification (Grill *et al*., 1985). PCs are synthesized from glutathione (GSH) by phytochelatin synthase (PCS) (Heiss *et al*., 2003). PCs are ubiquitous components in higher plants (Gekeler *et al*., 1989) and have also been found in algal species (Gekeler *et al*., 1988) and in some yeast, particularly *Schizosaccharomyces pombe*, in which they were first discovered (Murasugi *et al*., 1983). Here we describe the characterization of Cd-peptides complexes analyzed by gel-filtration chromatography in sunflower (*Helianthus annuus* L.) roots under Cd stress.

Materials and Methods

Growth Conditions

Sunflower (*Helianthus annuus* L.; var. LG 10-10) seeds were surface sterilized with 10% (v/v) H_2O_2 for 20 min, rinsed many times with tap water and germinated on filter paper moistened with distilled water in the dark, at 25°C for 3 days. The germinated seedlings were transferred to 12l basal nutrient solutions for 4 days. Selected plants of uniform size were selected and then transferred to identical solutions in 6l plastic pots (12 plants each) for 11 days. Fifteen-day-old seedlings were transferred to a fresh nutrient solution. Cd was added as Cd $(NO_3)_2$ at 75 µM. Control plants were placed in solution without Cd. The composition of the basal nutrient solution is according to Chaffai *et al*. (2005). At the end of the 3-d Cd-stress period, the plants were divided into different portions: leaves, stems and roots, the fresh weight measured and immediately frozen in liquid nitrogen. For dry weight determination, the plant material was desiccated at 70°C for 72 h. Cd toxicity was determined by measuring the plant biomass production, the root and stem length and the leaf area.

Gel-filtration Chromatography of Cd-binding Complexes

For extraction of total soluble protein, frozen (N_2) root material was ground in 20 mM Tris-HCl, pH 7.4 that contain 0.5 mM ascorbic acid (1 mL g tissue) and centrifuged for 25 min at 16 500 g at 4°C (Beckman Allegra 64R). The supernatant was loaded onto a 72×1.5 cm Sephadex G-50 (Pharmacia Fine Chemicals AB Uppsala, Sweden) column, equilibrated with protein extraction buffer (see above). The separation process depends on the different abilities of the various proteins to enter either, some, all or none of the beads, which in turn relates to the size of this protein. Fractions (4.5 mL) were collected and assayed directly for reduced sulfhydryl groups (-SH), Cd and Cd-peptides complexes. The elution volume (V_e) of a protein is determined by the size of the protein such that there is a logarithmic relationship between protein molecular mass and elution volume. The G-50 Sephadex column was calibrated with a range of globular proteins of known relative molecular mass (M_r): ovalbumin (68 kDa), bovine pancreas trypsine (24 kDa) and ribonuclease (13.7 kDa). An approximately linear calibration line is obtained by plotting a graph of log M_r versus K_d or V_e for the calibrating proteins. K_d is calculated from the following equation:

$$K_d = \frac{(V_e - V_0)}{(V_t - V_0)}$$

where V_0 is the volume in which molecules that are wholly excluded from the column material emerge (the excluded volume V_0= 58.5 mL), V_t is the volume in which small molecules that can enter all the pores emerge (the included volume V_t= 144 mL) and V_e is the volume in which the marker protein elutes. The construction of a calibration curve enables the M_r of the Cd-peptide to be estimated.

Measurement of non Protein Thiols (NPTs)

Total non protein thiols (NPTs) in the elution fractions were quantitated spectrophotometrically at 412 nm according to the method of Ellman (1959). An aliquot (0.5 mL) of the indicated fractions was added to 2 mL of sulfhydryl reaction buffer (0.25 M Tris-HCl, adjusted to pH 7.5). The reaction was initiated by adding 40 µL of a 10 mM sulfhydryl-reactive reagent dinitrobenzoic acid (DTNB) solution. After a 20 min reaction period, absorbance values of aliquots of the assay solution were measured at 412 nm. DTNB reacts with reduced sulfhydryl groups (-SH) to form a yellow nitromercaptobenzoic acid anion product. NPTs and PCs (NPTs-GSH) levels are expressed as µmoles of thiol equivalents per gram fresh wt.

Determination of Cadmium

Cadmium levels were measured in each 4.5 mL elution fraction by atomic absorption spectrophotometer (Perkin-Elmer 2380). The Cd-S thiol bond-specific absorbance (A_{254}) was measured using a UV-Visible spectrophotometer (Jenway 6105). The protein in the indicated fractions was quantified by reading the absorption at protein absorption maxima in the near-UV region at 280 nm. The Cd concentration in roots was estimated from the Cd concentration in the protein extracts.

Quantitation of GSH by HPLC

GSH was extracted by homogenizing root tissues in 10% (w/v) 5-sulfosalicylic acid (SSA) buffer solution (1 mL g tissue), using a mortar and pestle. The homogenate was centrifuged for 1 min at 10000 g at 4°C to remove cellular debris and precipitated proteins. GSH was separated from proteins by G-25 size-exclusion Sephadex chromatography. The supernatants were filtered over 0.45 µm filters and applied on a 1.6×9 cm Sephadex G-25 column, eluting with 100 mM sodium acetate and 10 mM NaCl. The collected fractions (1 mL) were used for GSH analysis. Samples (20 µL) were injected to a reversed phase-C_{18} column (0.5 µm, 15×4.6 ID, HP) and connected to an HPLC pump and the column was eluted isocratically with 17% acetonitrile and 0.01% phosphoric acid for 15 min at flow rate of 1 mL min^{-1}. The column was allowed to re-equilibrate in elution solvent for 10 min before the next injection. Retention times and peak areas were determined with a computerized integration program (HP Chemstation 4.0 for liquid chromatography) and peaks were detected at 210 nm. The GSH content were expressed as µmoles of thiol equivalents per gram fresh weight, using GSH as standard. Lyophilized phytochelatins: GSH, PC2, PC3 and PC4 (kindly provided by M.H Zenk, Bizentrum Universität-Halle-Germany) were used for HPLC calibration.

Results

Effect of Cd on Growth Parameters

When the sunflower seedlings were grown on nutrient medium in the presence of 75 µM Cd, growth was inhibited, the leaves became progressively chlorotic and roots showed a slightly brownish color. Chlorosis, mainly of younger leaves and sometimes cotyledon leaves appeared just 24 h after the treatment. The root, stem and leaf fresh wt. of the seedlings exposed to Cd decreased by 61.5, 75.7 and 58.8%, respectively (Table 1). The dry wt. of these organs was similarly reduced by about 52% (Table 1). The water content decreased by 20.1, 51.7 and 16.6%, respectively in roots, stems and leaves (Table 1). As shown in Table 2, root length was reduced by 37% compared with the control plant, but the stem length was slightly affected. The leaves of Cd-treated plants showed less leaf expansion than in the control plants, as indicated by a reduction in leaf area (Table 2).

Gel-filtration Chromatography of Total Proteins and Cd-binding Complexes

Absorbance at 254 and 280 nm of the chromatographic fractions eluted on Sephadex G-50 is shown in Fig. 1. The absorbance profiles at 254 and 280 nm showed two predominant peaks P_1 and P_2, eluted respectively at V_0 and V_t (fractions 13 and 32, respectively) of the column. In the A_{254} profile, the roots of Cd-treated plants revealed new peak (black arrow) at fraction 25. This peak which

Table 1: Effects of Cd on fresh weight, dry weight and water content in roots, stems and leaves of sunflower (*Helianthus annuus* L.) seedlings. Fifteen-day-old seedlings were transferred to nutrient medium containing 75 µM Cd $(NO_3)_2$. Values are means±SE of five (n = 5) plants determined after 3 days of Cd exposure. Asterisks show statically different means between control and treated plants: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

	Fresh weight (g)		Dry weight (mg)		Water content (g g^{-1} DW)	
	Control	Cd	Control	Cd	Control	Cd
Roots	0.87±0.10	0.33±0.09**	38.31±1.82	18.24±5.47**	21.62±1.96	17.27±1.52
Stems	1.45±0.16	0.35±0.06***	89.19±16.22	42.16±9.73*	15.27±1.90	7.37±1.51*
Leaves	1.20±0.09	0.49±0.12**	129.73±15.14	62.70±17.30*	8.25±0.52	6.88±0.58

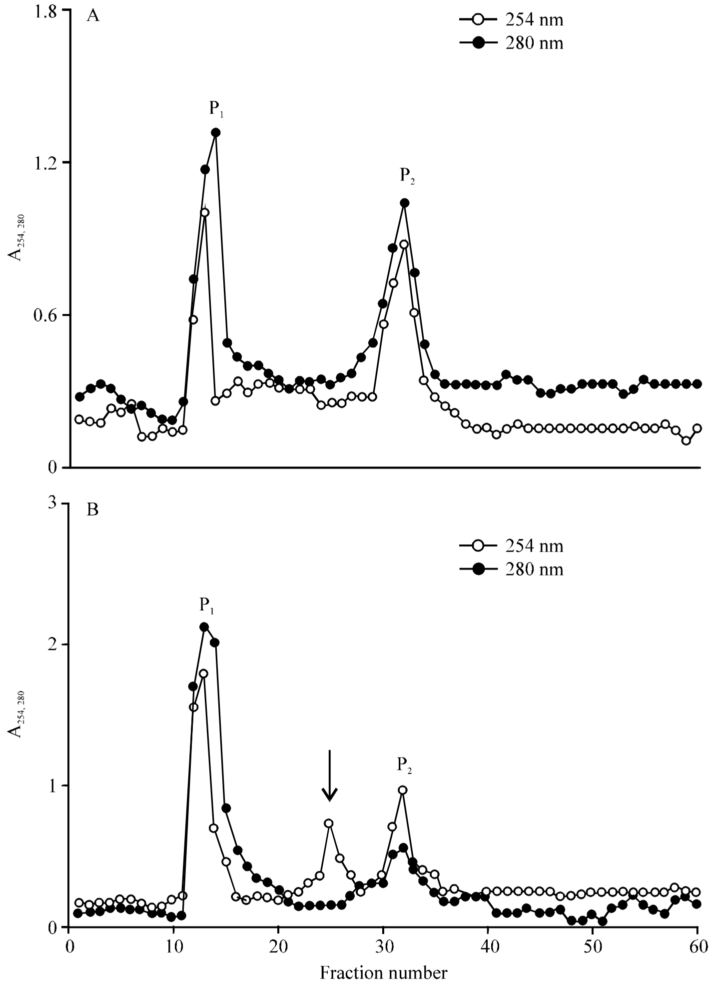

Fig. 1: Gel-filtration chromatography of total protein extracts from roots of sunflower (*Helianthus annuus*) seedlings exposed to 75 µM Cd in hydropnic culture for 3 days. Extracts were chromatographed on a Sephadex G-50 column. The excluded volume (V_0) peak is centered at fraction 13 (P_1), peak for the Cd-binding peptide is centered at fraction 25 (black arrow) and the included volume peak (V_t) is centered at fraction 32 (P_2). A, extracts of control plants; B, extracts of Cd-treated roots exposed to 75 µM Cd

Table 2: Effect of Cd on root length, stem length and leaf area of sunflower (*Helianthus annuus* L.) seedlings. Fifteen-day-old seedlings were transferred to nutrient medium containing 75 μM Cd $(NO_3)_2$. Values are means±SE of five (n = 5) plants determined after 3 days of Cd exposure. Asterisks show statically different means between control and treated plants: *, $p<0.05$; **, $p<0.01$

Cd (μM)	Root length (cm)	Stem length (cm)	Leaf area (cm^2)
0	27.69±2.05	26.15±0.51	20.36±1.81
75	17.44±1.54**	23.59±0.48**	14.31±1.41*

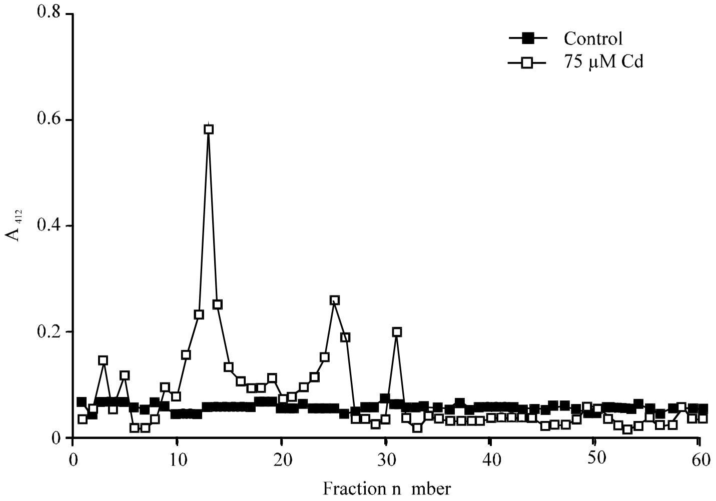

Fig. 2: Gel-filtration chromatography of total protein extracts in sunflower roots on Sephadex G-50 column. The specific binding of Cd to -SH groups was determined by absorbance at 412 nm. The S-Cd complex is centered at fraction 25

absorbs more at 254 nm than in 280 nm ($A_{254}/A_{280} > 1$) may correspond to Cd-binding peptides. In addition, the peak eluted at V_0 was slightly increased by the Cd treatment and was probably due to nonspecific binding of Cd to cellular components. As seen in Fig. 2, the elution profiles of total -SH groups indicated that the Cd-peptide complex having high absorbance at 412 nm (eluting at fraction 25), is rich in -SH groups. The-SH peak induced by Cd is absent in roots of control extracts (Fig. 2). This peak is designated PC-Cd complex. The concentrations of Cd in the chromatographic fractions eluted on Sephadex G-50 in the Cd-treated roots revealed that almost all Cd eluted and coincided with PC-Cd complex eluted at fraction 25 (Fig. 3). The detected PC-Cd complex has an apparent molecular mass of 17.8 kDa (Fig. 4).

Accumulation of Cd, NPTs and GSH in the Control and Cd-treated Roots

The effect of excess Cd (75 μM) on total non protein thiols (NPTs), glutathione (GSH) and phtochelatins (PCs) determined as NPTs minus GSH are shown in Table 3. There was 14.3% decrease in the GSH level by the metal treatment, compared to the control. The total NPTs (1.54 μmol g^{-1} fresh wt.) represent approximately the GSH concentrations (1.40 μmol g^{-1} fresh wt.) in the control plants, resulting in lower PCs levels (0.14 μmol g^{-1} fresh wt.). However, the concentrations of the NPTs increased 3.6-fold in Cd-treated roots. The PCs levels in root extracts from Cd-treated seedlings were 39-fold of control-plant levels (Table 3). In addition, Cd accumulated at high levels in Cd-treated sunflower roots (Table 3). The PC:Cd ratio determined in root extracts of Cd-stressed plants has a value of 0.82 (Table 3).

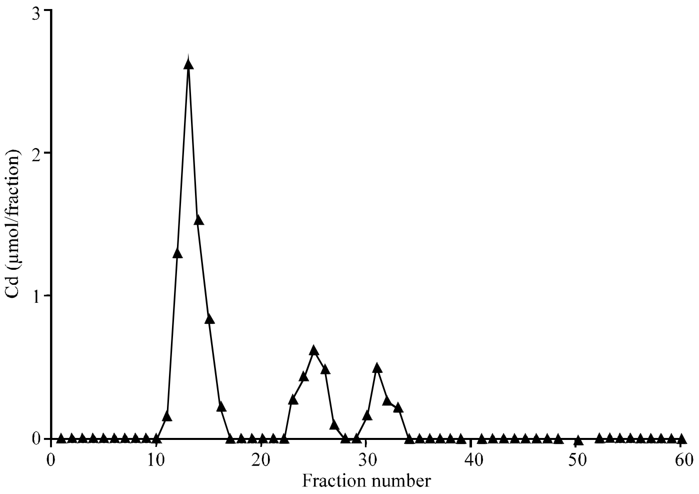

Fig. 3: Cadmium in the eluted fractions measured by an atomic absorption spectrophotometer. Total protein extract of sunflower roots was analyzed by gel-filtration chromatography on Sephadex G-50 column

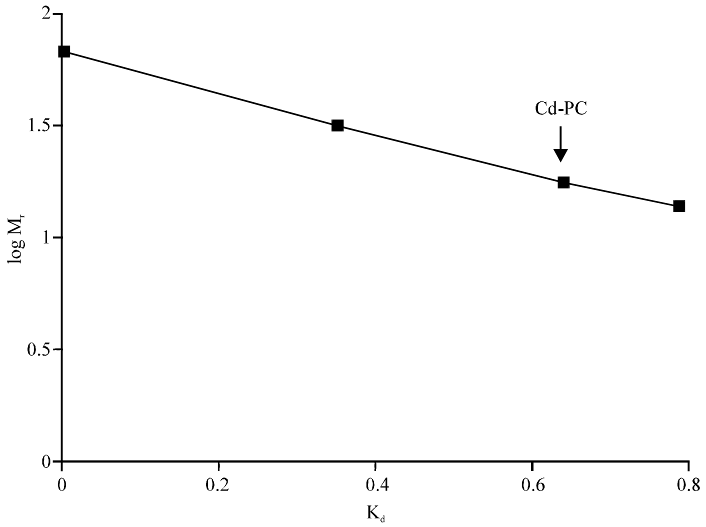

Fig. 4: A graph of log M_r versus K_d for the range of globular proteins of known relative molecular mass (M_r): ovalbumin (68 kDa), bovine pancreas trypsine (24 kDa) and ribonuclease (13.7 kDa). The relative molecular mass (M_r) of the PC-Cd complex detected by gel-filtration analysis is estimated to 17.8 kDa

Table 3: Total NPTs, GSH, PCs and Cd accumulation in roots of sunflower (*Helianthus annuus* L.) seedlings. Total NPTs, GSH, PCs (measured as NPTs-GSH) and Cd were determined as µmol g^{-1} fresh wt. GSH was analyzed by HPLC as described in Materials and Methods. Values are means±SE of three (n = 3) plants, ND: Not Detected

	Total NPTs	GSH	PC (NPTs - GSH)	Cd	Percentage of total thiol as PC	PC:Cd ratio
Cd (µM)	µmol g^{-1} fresh wt.					
0	1.54±0.03	1.40±0.02	0.14±0.08	ND	9.09	ND
75	5.53±0.04	1.20±0.04	5.41±0.04	6.63±0.03	97.83	0.82

Discussion

First, we examined the Cd effects on growth parameters of sunflower seedlings. Cd toxicity has been reviewed most extensively by Sanità di Toppi and Gabbrielli (1999). Numerous studies have shown that heavy metal stress affect the plant growth, which can depend at the same time on plant species and the metal concentrations in the growth medium (Borges and Wollum, 1981; Seliga, 1993). In a preliminary report, Di Cagno *et al*. (1999b) presented data confirming a very strong dependence of the growth of sunflower plants on Cd^{2+} concentration in the nutrient medium, which is reflected by the decrease in both leaf area and leaf fresh wt. It has been shown that clover plants grown in heavily polluted soil containing Cd showed reduced growth (McGrath *et al*., 1988). Similarly, Cd stress has been shown to cause a decrease in the dry matter of roots and aerial parts of *Alnus rubra* (Wickliff *et al*., 1980) and tomato (Chaffei *et al*., 2004). Similarly to our results, a reduced root length was observed in maize (Keltjens and Van Beusichem, 1998). Therefore, the root elongation seems to be hypersensitive to Cd toxicity, as reported by Hogan and Rauser (1981). The decrease in root length is considered to be a typical symptom for the heavy metal toxicity (Arduini *et al*., 1994). Damage to root system may be related to an increased Cd accumulation. In fact, the ability of roots to retain more Cd than the aerial parts is consistent with previous reports in several plant species, when tested in a hydroponic system. Cd toxicity may also result from deficiency of essential heavy-metals such as Fe, Zn, Mn, Mg, Ca or K. Inhibition of metal uptake has been interpreted as resulting from interactions between Cd and these essential nutrient elements (Sela *et al*., 1989). The results of experiments on nutrient uptake showed 80% decrease in the phosphate uptake by *Nostoc linkia* under Cd stress (Husaini and Rai, 1991). Moreover, plants exposed to increasing external Cd concentrations decreased accumulation of K^+ and Mg^{2+} in the roots, nodules and aerial parts (Borges and Wallum, 1981; Seliga, 1993). This study provides evidence for the inhibition of photosynthesis processes, as indicated by the decrease in the shoot growth parameters (biomass production, leaf area, stem length). For a long time, heavy metals have been proposed as having damaging effects on photosynthesis in algae and higher plants (Krupa *et al*., 1993). Indeed, Cd-induced alterations in photosynthetic activity have been noted previously at different levels: Cd has been reported to inhibit the photosynthetic electron transport in PSI and PSII (Husaini and Rai, 1991) and to decrease the chlorophyll (Baszyński *et al*., 1980) and cytochromes contents (Seliga, 1993). In the present study, we demonstrated that the water content of sunflower plants was decreased, which can had an effect on the transpiration process. In fact, the reduction in the water uptake or transport may lead to a reduction in the stomata opening (Hagemayer and Waisely, 1989) and therefore to decreased transpiration process (Haag-Kerwer *et al*., 1999).

It will be of further interest to examine the accumulation of Cd-binding complexes in sunflower roots in order to determine the mechanisms involved in Cd sequestration. The mechanisms of Cd tolerance have been thoroughly studied in plant cell cultures or intact plants. The gel-filtration analysis showed that single peak was detected in roots of Cd-treated plants and thus may represent Cd-thiolate (Cd-S) complex. The formation of Cd-thiolate complexes in phytochelatins (PCs) has been shown in response to Cd exposure (Strasdeit *et al*., 1991). The chromatograms showed two major peaks, eluted at V_0 and V_t fractions, respectively. A similar elution profile was reported by many studies (Leita *et al*., 1991). The eluted peak at the V_t and V_0 fractions consist of LMW (Kishinami and Widholm, 1987) and HMW Cd-binding material (Leita *et al*., 1991), respectively. In extracts of *Helianthus annuus* exposed to 75 µM Cd, the eluted peak at fraction 25 (Fig. 1B, black arrow) is designated PC-Cd complexes. This compound, which is induced by Cd has an apparent molecular mass of 17.8 kDa, as previously shown in *Silene vulgaris* (De Knecht *et al*., 1992). The thiol-peptides analysis at 412 nm (A_{412}) identified Cd induced peak eluted as above mentioned peak. Present results also indicated that most of the Cd eluted in the PC peak and strongly suggest that Cd-induced peptides production. These peptides with A_{254}/A_{280} ratio > 1 confirm the presence of -SH in Cd peptides complexes and support the involvement of these peptides in the defense mechanisms (Rauser, 1995; Inouhe *et al*., 2000). The relevance of peptides as effective chelators of the metal ions has been largely described by many authors (Cobbett, 2000). The non protein metal-binding peptides in selected Cd resistant tomato cells were found to sequestrate up to 80% of cellular Cd (Steffens *et al*., 1986). These peptides are named PCs (Grill, 1985). NPTs, of which PCs are the primary constituent (Harmens *et al*., 1993), showed the highest levels in Cd-treated sunflower roots attributed to increased levels of PCs. PCs play a role in essential heavy-metal homeostasis and are known to serve as the sulfur transport form in plants (Robinson, 1989). The significance of PCs for heavy metals sequestration has frequently been reviewed (Rauser, 1995; Zenk, 1996). These small heavy metal binding-peptides contributed to the protection from heavy metal toxicity in several plant species and in some fungi as well (Ishikawa *et al*., 1997). The PCs are the ligand-class that is predominantly produced by plants in the response to heavy metal stress such as Cu and Zn and have been particularly detected in response to Cd (Grill *et al*., 1985, 1987). PCs ensure efficient Cd detoxification by complexation and vacuolar sequestration (Grill *et al*., 1985; Cobbett, 2000). PCs are able to chelate Cd ions (Cd(II)), sequestering them in a non-toxic form (Steffens *et al*., 1986). The chelated metals are transported to the tonoplast, taken up by active transport systems and deposited in the vacuole (Tommasini *et al*., 1998). That PC play an important role in the detoxification of heavy metals and tolerance has been inferred from genetic studies and experiments using BSO of various mutants of *S. pombe*, in which a deficiency in the biosynthesis of PCs increased heavy metal sensitivity (Howden *et al*., 1995). In addition, genetic studies using *S. pombe* have shown that GSH deficient mutants are also PC deficient and Cd hypersensitive (Glaeser *et al*., 1991). In an experiment, where plant cells were grown in the presence of BSO, an inhibitor of GSH biosynthesis, PC production did not occur and result in more sensitivity of the cells to Cd (Mendum *et al*., 1990). It has been shown that Cd-tolerant tomato cells accumulated higher levels of PCs than the non tolerant line (Steffens *et al*., 1986). Moreover, Cu tolerance in a naturally selected line of *Mimulus guttatus* appears to be attributable to PC formation (Salt *et al*., 1989). Present results suggest that in roots, Cd induced a decrease in GSH level, confirming that GSH is a precursor for PC biosynthesis (Grill *et al*., 1989). The GSH is an important component of heavy-metal detoxification processes, ascorbate-glutathione cycle and serves as the transport form of the reduced sulfur (May *et al*., 1998). Thus, the ability to synthesize GSH appears to be crucial for protection from Cd, as shown by the increased tolerance of plants with elevated levels of GSH and a decreased tolerance in plants with diminished levels of GSH (Howden *et al*., 1995). As can be inferred from this study, the GSH shift is a common response to Cd for phytochelatin production (Zenk, 1996). The GSH level has been shown to be sensitive to environmental factors such as light intensity (Noctor *et al*., 1997), herbicides (Foyer *et al*., 1995) and heavy metals (Schneider and Bergmann, 1995). Exposure to heavy metals initially resulted in a severe depletion of GSH in many plant species (Cd: *Rauvolfia serpentine*: Grill *et al*., 1987; pine: Schützendübel *et al*., 2001; carrot: Sanità di Toppi *et al*., 1999; tobacco: Vögeli-Lange and Wagner, 1996; Cu: *Silene cucubalus*: de Vos *et al*., 1992; Cu or Cd: *Arabidopsis*: Xiang and Oliver, 1998; Ni and Zn: pigeonpea: Rao and Sresty, 2000; Fe, Cu or Cd: sunflower leaves: Gallego *et al*., 1996).

In conclusion to this study, it seems that accumulation of PCs is a major component of Cd-detoxification process in roots of sunflower plants, but several other mechanisms may also operate.

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