

Subcellular Localization of Cadmium and Cadmium-Binding Peptides in Tobacco Leaves¹

Implication of a Transport Function for Cadmium-Binding Peptides

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ABSTRACT

The synthesis of Cd-binding peptides (CdBPs) was induced upon addition of 20 micromolar CdCl₂ (nonphytotoxic level) to the nutrient solution of hydroponically grown tobacco seedlings (*Nicotiana rustica* var Pavonii). Amino acid analysis showed that the main components were γ -(Glu-Cys)₂-Gly and γ -(Glu-Cys)₄-Gly. Seedlings exposed to the metal for 1 week contained similar glutathione levels as found in the controls (about 0.18 micromole per gram fresh weight). If, as has been proposed, CdBPs are involved in Cd-detoxification by chelation, both metal and ligand must be localized in the same cellular compartment. To directly determine the localization of Cd and CdBPs, protoplasts and vacuoles were isolated from leaves of Cd-exposed seedlings. Purified vacuoles contained virtually all of the CdBPs and Cd found in protoplasts (104% \pm 8 and 110% \pm 8, respectively). CdBPs were associated with the vacuolar sap and not with the tonoplast membrane. Glutathione was observed in leaves and protoplasts but not in vacuoles. The probability that CdBPs are synthesized extracellularly and our finding that they and Cd are predominantly located in the vacuole suggest that these molecules might be involved in transport of Cd to the vacuole. Our results also suggest that a simple cytoplasmic chelator role for CdBPs in Cd tolerance cannot be assumed.

Various mechanisms have been proposed for detoxification of potentially toxic heavy metal ions in plants. Metal ions such as Cd (also Hg, Pb, and Cu) can interfere with the plant's metabolism by binding to essential sulfhydryl groups of enzymes or structural proteins. Therefore, it seems necessary to keep the intracellular concentration of potentially toxic heavy metal ions at a low level. This can be achieved by a number of different mechanisms (31). In Cd-resistant *Euglena gracilis*, for instance, decreased metal uptake has been proposed as the principal mechanism of metal tolerance (1). Extracellular sequestration of Cd and active metal efflux have been reported for some bacteria (18, 32). It is not known if similar mechanisms function in higher plants. Rather than export or extracellular sequestration, compartmentation in the vacuole has

been suggested as a means of metal detoxification in higher plants (34). Another mechanism of metal tolerance involves the chelation of heavy metals by specific organic molecules, thereby lowering the intracellular concentration of potentially toxic free metal ions. A possible involvement of organic acids in detoxification of heavy metals was first discussed by Ernst (see 34). We have studied this aspect in recent work (14). Particularly in the case of Cd toxicity, however, most attention has centered on cysteine-rich γ -glutamyl peptides called CdBPs or phytochelatins which are highly induced by Cd and bind it with high affinity (8, 21, 25, 28). In contrast to metallothioneins, which are considered to be a principal means for metal detoxification in animal systems and some fungi (31), CdBPs are not primary gene products (24). They are structurally related to GSH and their biosynthetic pathway probably involves GSH or metabolites of GSH.

Several authors (8, 22, 25, 28) have reported positive correlations between the occurrence of CdBPs and tolerance to Cd. If chelation of Cd by CdBPs in the cytosol is a principal mechanism of tolerance, then the bulk of both metal and ligand must be localized in this compartment. Current evidence concerning the subcellular localization of Cd is not entirely consistent. Rauser and Ackerley (20) found Cd associated with electron dense granules in the cytoplasm, vacuoles and nuclei of *Agrostis* and maize roots whereas Khan *et al.* (12), also using maize roots, reported on the presence of Cd only in the walls of sieve elements and the middle lamella separating the endodermis from the pericycle. X-ray microanalysis revealed that in the marine alga *Dunaliella bioculata* treated with high levels of Cd, the metal was exclusively localized in the vacuole (10). In another study involving the aquatic fern *Azolla filiculoides* high concentrations of Cd were found by x-ray microanalysis in aggregates with phosphate and calcium. Most of these aggregates were detected in cell walls of bundle cells and xylem cells of the bundle (27). To our knowledge, no published information is currently available concerning the subcellular localization of CdBPs.

Here, we address the question whether chelation of Cd by CdBPs is an important mechanism of resistance by directly determining the subcellular localization of Cd and CdBPs in protoplasts and vacuoles isolated from leaves of Cd-exposed tobacco seedlings (*Nicotiana rustica* var Pavonii). We demonstrate that virtually all of the Cd and CdBPs are localized

¹ Abbreviations: CdBPs, Cd-binding peptides; Chaps, 3-[(3-cho-lamidopropyl)-dimethylammonio]1-propanesulfonate; SH, sulfhydryl; UDP-glucose ST, UDP-glucose sterol glucosyltransferase.

in the vacuole in tobacco seedlings grown in the presence of 20 μM Cd.

MATERIALS AND METHODS

Plant Material

Tobacco seeds (*Nicotiana rustica* var *Pavonii*) were germinated on vermiculite in Eppendorf tubes in quarter strength Hoagland solution. The last 7 mm of Eppendorf tubes were removed to allow for the roots to grow into the nutrient solution. After 14 d, three seedlings each were transferred to 450 mL styrofoam beakers also containing quarter-strength Hoagland solution. Thereafter, the nutrient solution was changed weekly. Cd exposure was started 21 d after the initiation of germination by adding CdCl_2 to the nutrient solution to achieve a 20 μM concentration. Tobacco seedlings were kept at 23 to 25°C with continuous illumination by cool white fluorescent light ($\sim 300 \mu\text{E m}^{-2}\text{s}^{-1}$). Experiments were performed with leaves from 26 to 30 d old control and Cd-exposed tobacco seedlings. Typically, leaves 7 and 8, counted from the bottom of the plant, were used for either acid extraction or protoplast and vacuole isolation.

Assay for GSH and CdBPs

Acidic leaf extracts were prepared from fresh leaves without midribs by homogenizing 250 mg (fresh weight) leaf material in 750 μL 6.67% (w/v) sulfosalicylic acid (25) at 4°C using a mortar and pestle. The homogenate was transferred to Eppendorf tubes, centrifuged at 16,000g for 5 min, and the acid soluble supernatant was analyzed immediately for total non-protein SH, GSH, and CdBPs. Total nonprotein SH was determined by the method of Scheller *et al.* (25). GSH and CdBPs were analyzed by HPLC using a system similar to that described by Grill *et al.* (8). Briefly, low mol wt thiols were separated on a C_{18} reverse phase column (Waters/Millipore, part No. 086344) using a 0 to 20% acetonitrile gradient in 0.05% (v/v) phosphoric acid over 20 min. On-line, postcolumn derivatization (Applied Biosystems, Ramsey, NJ, 520 Post Column Reactor) with Ellman's reagent was used to specifically monitor sulfhydryl containing compounds. The reagent consisted of 120 mM Na-phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM 5,5'-dithiobis(2-nitrobenzoic acid), and detection was at 412 nm. Flow rates for solvent and reagent were 1.0 mL/min. For leaf extracts, an aliquot corresponding to 5 mg fresh weight was injected immediately after addition of 1 nmol *N*-acetyl cysteine as internal standard. The addition of *N*-acetyl cysteine was omitted when comparing leaves, protoplasts, and vacuoles, because purified vacuoles rapidly hydrolyzed this substrate. Protoplasts and vacuoles were lysed by adding 10 μL of 66.7% (w/v) sulfosalicylic acid to 500 μL sample. After centrifugation at 16,000g for 5 min the acid soluble supernatant was subjected to analysis by HPLC. Amounts of GSH and CdBPs were calculated from a standard curve using GSH (Sigma Chemical Co. No. G-4251) as a standard.

Preparation and Purification of Protoplasts

To prepare mesophyll protoplasts, the lower epidermis was removed by peeling and the leaves were cut into approxi-

mately 1 cm^2 pieces. Protoplasts were liberated by placing 0.7 to 1.0 g leaf material (fresh weight) peeled side down in Petri plates of 8 cm diameter containing 10 mL 2.0% (w/v) Cellulysin (Calbiochem Co.), 0.5% Pectinase (72 units/mL Sigma Chemical Co. No. P-5146) a solution of which was desalted on Sephadex G-25 and then made 0.3 M with mannitol. Incubation was for 1 to 1.5 h at 29°C with gentle shaking. The suspension then was filtered through a nylon net (mesh size 25 μm), rinsed with 3.0 mL 0.3 M mannitol, and layered onto 4.0 mL 15% (w/v) Ficoll in 0.3 M mannitol. After centrifugation for 4 min at 150g intact protoplasts were collected from the interphase and diluted with 8 mL 0.3 M mannitol. The protoplast suspension was again layered on a cushion of 15% (w/v) Ficoll in 0.3 M mannitol and after centrifugation under the same conditions purified protoplasts were recovered from the interphase.

Preparation and Purification of Vacuoles from Mesophyll Protoplasts

Vacuoles were isolated using a modification of the method of Matoh *et al.* (17). Lysis of protoplasts was induced by mixing 0.5 mL protoplasts (approximately 1.0×10^6 protoplasts/mL) with 10 mL vacuole isolation medium consisting of 0.18 M mannitol, 1.0 mM EGTA, 0.5 mM Chaps (Sigma Chemical Co.), and 20 mM Hepes (pH 8.0) (adjusted with 1 M Tris solution). The mixture was gently stirred with a wooden dowel. During this period (5 min) more than 90% of the protoplasts lysed. The lysate was divided into two portions and each was mixed with an equal volume of 20% (w/v) Ficoll in 0.24 M mannitol, 1.0 mM EGTA, 0.5 mM Chaps, and 20 mM Hepes-Tris (pH 8.0) to make the lysate 10% (w/v) with Ficoll. This was overlaid with 3.0 mL 3.9% Ficoll and 1.5 mL 0% w/v Ficoll containing the same components as the 20% (w/v) Ficoll solution with the exception of EGTA which was omitted from the upper layers. After 15 min of centrifugation at 150g most of the vacuoles floated to the 0/3.9% Ficoll interphase. Vacuoles were collected with a pasteur pipette.

Separation of Tonoplast and Vacuolar Sap

Vacuoles were disrupted by sonication in a bath sonifier and subsequently frozen at -80°C . After thawing, tonoplast membranes were sedimented by centrifugation at 100,000g for 1 h. The supernatant was removed and the membranes were washed in a small volume of 0.24 M Mannitol, 0.5 mM Chaps, 20 mM Hepes-Tris (pH 8.0), and sedimented again under the same conditions. The acidified supernatant and acid extractable components of the pellet were immediately assayed for CdBPs by HPLC.

Tissue Extracts

Tissue extracts for total protein determinations were prepared by homogenizing leaf material without midribs in 5 mL/g fresh weight of 25 mM KPO_4 (pH 7.4), 10 mM β -mercaptoethanol using a mortar and pestle. Homogenates were clarified by centrifugation at 23,500g for 10 min and the supernatant was used for protein determinations.

Enzyme Assays

α -Mannosidase was measured in an assay mixture containing 0.2 mL 0.1 M citrate-NaOH (pH 5.0), 0.1 mL 5 mM *p*-nitrophenyl- α -D-mannopyranoside and 0.05 to 0.2 mL extract to a total volume of 0.5 mL. Incubation was at 39°C for 1 to 2 h, the assay was stopped by adding 0.5 mL 0.2 M borate-NaOH (pH 9.8) and read at 400 nm. Chl was estimated by the method of Strain *et al.* (30). Malate dehydrogenase was measured according to Worthington (35). UDP-glucose ST was determined by the method of Hartmann-Bouillon and Benveniste (9). Cyt *c* oxidase was assayed as described by Hodges and Leonard (11), and protein was determined by the method of Bradford (4) using BSA as a standard.

Other Analytical Procedures

Cadmium was measured by atomic absorption spectroscopy after samples were made 1 N with HCl. Performic acid oxidation and amino acid analysis was as described in Reese and Wagner (21).

RESULTS

Leaves of tobacco seedlings treated with 20 μ M CdCl₂ for 1 week showed no morphological signs of Cd-toxicity, and there was no significant difference in fresh weight between control and Cd-treated leaves. Similar results were found when the nutrient solution was changed daily to maintain a Cd-concentration of 20 μ M CdCl₂ (data not shown). Also, total protein and Chl contents were similar in leaves from control and Cd-treated seedlings (Table I). However, after exposure to Cd, the total nonprotein SH content increased markedly from 0.19 μ mol SH/g fresh weight to 1.23 μ mol SH/g fresh weight. HPLC analysis of acidic leaf extracts from control and Cd-treated seedlings showed that GSH levels were similar in control and Cd-treated leaves, but addition of 20 μ M CdCl₂ to the nutrient solution resulted in the appearance of four additional peaks (Fig. 1). These Cd-induced, SH components were designated peaks 1 to 4. Summation of SH components of Figure 1, A or B, and comparison of total SH recovered with that injected showed recovery to be \geq 90%. Therefore, the increase in nonprotein SH (Table I) resulting from Cd-exposure can be attributed to Cd-induced peaks 1 to 4 (Fig. 1A). To determine if small peaks observed in control leaves in the region of components 2 to 4 were different from those occurring after Cd induction, extracts from control and Cd-treated leaves were mixed and analyzed by HPLC. With the possible exception of the component eluting with a retention time of 14.47 min, components found in control extracts were

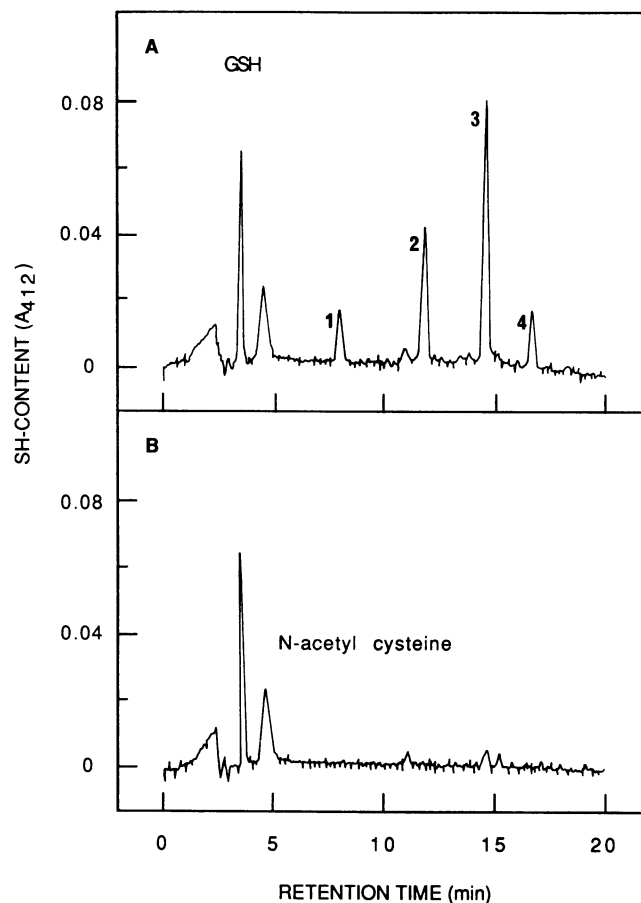


Figure 1. HPLC separation of low mol wt thiols from acidic leaf extracts of Cd-treated (A) and control (B) tobacco seedlings. Aliquots corresponding to 5 mg fresh weight were injected. Detection of thiols was at 412 nm after postcolumn derivatization with Ellman's reagent. Seedlings were 4 weeks old and treatment with 20 μ M CdCl₂ was for 1 week.

clearly distinct from those induced by Cd (data not shown). The induction of CdBPs in response to Cd exposure has previously been reported for many other whole plants and plant cells in culture (8, 21, 25, 28, and refs. therein). Grill *et al.* (8) obtained similar HPLC profiles after analysis of extracts from Cd-treated plant material, and Cd-induced, SH containing components were shown to be CdBPs (called phytochelatins).

Purification and Characterization of CdBPs from Tobacco Leaves

To establish that the Cd-induced sulfhydryl containing components were CdBPs, large scale preparations were made to determine the molecular composition of the sulfhydryl components. Purification of CdBPs from tobacco leaves involved acid extraction in 6.67% (w/v) sulfosalicylic acid followed by neutralization of the acid soluble supernatant by adding Tris crystals. Neutralization of the acidic extract was necessary to allow binding of Cd to the peptides (21). The neutralized extract was centrifuged at 23,500g and 4°C for 10 min and the supernatant was fractionated on a Sephadex G-

Table I. Protein, Chl, and Nonprotein SH Content in Leaf Extracts of 4 Week Old Tobacco Seedlings

Values represent the means and SE of duplicate samples from three independent experiments.

Treatment	mg/g fresh wt		μ mol/g fresh wt
	Protein	Chl	
Control	15.05 \pm 2.07	1.44 \pm 0.12	0.19 \pm 0.05
20 μ M CdCl ₂	19.04 \pm 3.08	1.47 \pm 0.16	1.23 \pm 0.14

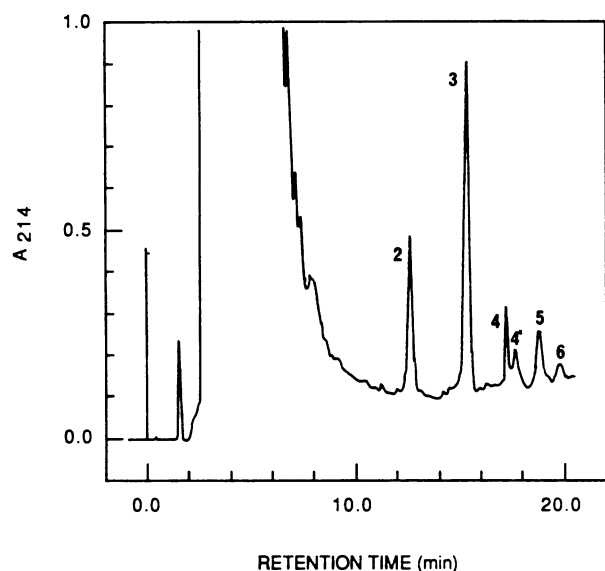


Figure 2. Chromatogram of purified CdBPs from tobacco leaves after acidic extraction, gel-filtration on Sephadex G-50, and lyophilization. Detection was in the UV at 214 nm. Postcolumn derivatization with Ellman's reagent was omitted to check purity of material and to collect samples for amino acid analysis.

Table II. Amino Acid Composition of Purified CdBPs from Tobacco Leaves

Amino Acid	Amino Acid Composition (Residue %)				
	peak 2	peak 3	peak 4	peak 5	peak 6
Cys	38.0	38.0	37.7	39.9	35.0
Asp/Asn	0.2	0.1	0.0	0.0	0.0
Thr	0.3	0.2	0.1	0.0	0.0
Ser	0.3	0.3	0.1	0.0	0.0
Glu/Gln	46.2	49.5	48.5	48.6	53.0
Pro	0.0	0.0	0.0	0.0	0.0
Gly	14.2	11.4	10.5	11.1	11.9
Ala	0.3	0.2	0.4	0.1	0.0
Val	0.0	0.0	0.4	0.1	0.1
Ile	0.1	0.0	0.1	0.0	0.0
Leu	0.1	0.0	0.1	0.0	0.0
Tyr	0.0	0.0	0.0	0.1	0.1
Phe	0.0	0.0	0.0	0.0	0.1
His	0.0	0.0	0.0	0.0	0.0
Lys	0.0	0.1	0.0	0.0	0.0
Arg	0.4	0.2	2.3	0.0	0.0
<i>n</i>	3	4	4	4	4

50 column (1.5 × 92 cm) equilibrated and eluted with 50 mM (NH₄)₂CO₃ buffer (pH 7.5). CdBPs were monitored by ¹⁰⁹Cd exchange binding as described in Reese and Wagner (21). ¹⁰⁹Cd containing fractions were pooled and freeze-dried. Further purification was achieved by HPLC as described in "Materials and Methods" with the exception that the postcolumn derivatization step was omitted and peptides were monitored directly at 214 nm. As shown in Figure 2, CdBP peaks 2, 3, and 4 were well separated and free of contaminating material. CdBP peak 1, only a minor component in our system, was masked by UV-absorbing material. In addition

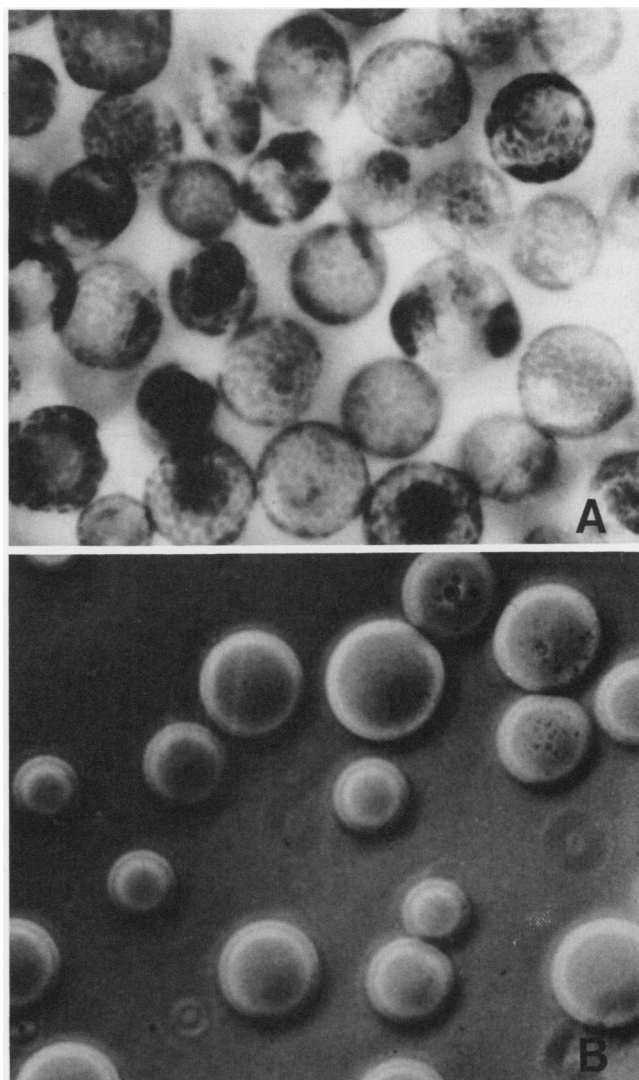


Figure 3. Purified protoplasts (A) and vacuoles (B) from leaves of 4 week old tobacco seedlings treated with 20 μM CdCl₂. Note in (A) the even distribution of chloroplasts characteristic of healthy protoplasts (brightfield microscopy). In (B) vacuoles were viewed using Nomarski differential interference contrast microscopy to evaluate the degree of surface contamination.

to the usual four Cd-induced peaks, small peaks (peaks 4', 5, and 6, respectively) occurred as a result of lyophilization or some other artifact of large-scale, multistep preparation. Amino acid analysis (Table II) showed that the predominant components in our system, peaks 2 and 3, consisted only of three different amino acids, cysteine, glutamate/glutamine, and glycine. The amino acid composition and virtual absence of other amino acids attests to purity and identity of these components. From the molar ratio of cysteine and glutamate/glutamine to glycine *n*-numbers were calculated and peaks 2 and 3 were identified as γ-(Glu-Cys)₃-Gly and γ-(Glu-Cys)₄-Gly, respectively. CdBP peaks 4 to 6 also represented molecules with a *n*-number of four. One possible explanation for the separation of chemically identical molecules by HPLC is that these molecules may represent charge isomers, given that

Table III. Level of Marker Enzymes, Chl, and Protein in Protoplasts and Vacuoles from Tobacco Leaves

Values represent the means of duplicate samples from at least two experiments.

Marker	Level per 10 ⁶ Protoplasts	Level per 10 ⁶ Vacuoles	% in Vacuole
α -Mannosidase (nmol/min)	2.16	2.19	101.4
NAD-Malate dehydrogenase (nmol/min)	6450	11.3	0.18
Cyt <i>c</i> oxidase (nmol/min)	104	ND ^a	0
UDP-glucose ST (cpm/h)	3399	ND	0
Chl (μ g)	247	ND	0
Protein (μ g)	2200	51.9	2.36

^a Not detectable.

Table IV. Vacuolar/Extravacuolar Distribution of Cd-binding Peptides and Cd in Tobacco Leaves

Experiment	Protoplasts	Vacuoles	% in Vacuole	
(nmol SH/nmol <i>p</i> -nitrophenol min ⁻¹) ^a				
CdBPs	I	16.8	16.0	95
	II	38.9	43.2	111
	III	29.9	31.9	107
$\bar{X} = 104 \pm 8$				
(nmol Cd/nmol <i>p</i> -nitrophenol min ⁻¹)				
Cd	I	6.6	7.7	112
	II	18.8	18.9	101
	III	9.5	11.1	117
$\bar{X} = 110 \pm 8$				

^a nmol SH represent total SH of Cd-binding peptide peaks 2, 3, and 4; GSH was used as a standard for SH and α -mannosidase activity was measured with *p*-nitrophenyl- α -D-mannopyranoside as substrate.

Table V. Distribution of CdBPs between Vacuolar Sap and Pellet

Fraction	CdBPs per 10 ⁶ Vacuoles	
	nmol SH ^a	
Intact vacuoles	67.43	70.39
100K g supernatant	92.86	75.65
100K g pellet	ND ^b	ND

^a Results obtained from two independent experiments; nmol SH represent total SH of Cd-binding peptide peaks 2, 3, and 4; GSH was used as standard for SH. ^b Not detectable.

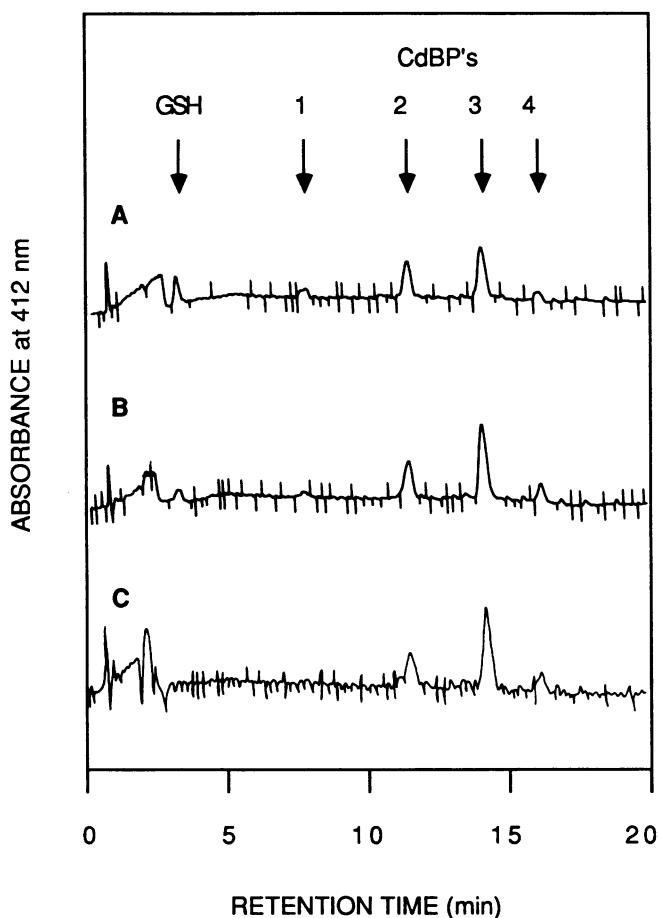


Figure 4. HPLC separation of low mol wt thiols from (A) leaves (approximately 0.5 mg fresh wt), (B) protoplasts, and (C) vacuoles of tobacco seedlings grown for 1 week in the presence of 20 μ M CdCl₂. Each sample represents equivalent amounts of α -mannosidase activity (1.4 nmol *p*-nitrophenol/h).

the pKs of the α -carboxyl of glycine and glutamic acid are 2.34 and 2.19, respectively, and the pH of the eluting solvent is 2.8.

Protoplast and Vacuole Isolation

A typical preparation of protoplasts and vacuoles from Cd-exposed tobacco leaves is shown in Figure 3. One g leaf material yielded about 1 to 2 $\times 10^6$ protoplasts which accounted for approximately 40% of the Chl content from leaves. Using α -mannosidase as a marker for vacuoles, vacuole yields were determined to be 10 to 20%. α -Mannosidase, as first shown by Boller and Kende (3), is often used as a marker enzyme for the vacuolar sap. In order to determine whether this is also the case for tobacco leaves, we compared α -mannosidase activities of purified protoplasts and vacuoles. Based on the microscopic observation that one protoplast releases one vacuole, we concluded that all of the observed α -mannosidase activity is localized in the vacuole in this tissue (Table III). Thus, α -mannosidase activity could serve as a marker for quantifying vacuolar/extravacuolar distributions.

Purity of Vacuole Preparations

As evaluated by microscopic analysis, protoplasts were virtually absent from vacuole preparations. Marker enzyme analysis (Table III) showed that vacuoles prepared from tobacco mesophyll protoplasts contained very little NAD-malate dehydrogenase activity, a marker to contamination with extravacuolar (cytosol, etc.) soluble components. The membrane marker Chl, though abundant in protoplasts, was absent from purified vacuoles. Activities of the other two membrane markers, Cyt *c* oxidase (mitochondrial inner membrane) and UDP-glucose ST (plasma membrane) were not detected in

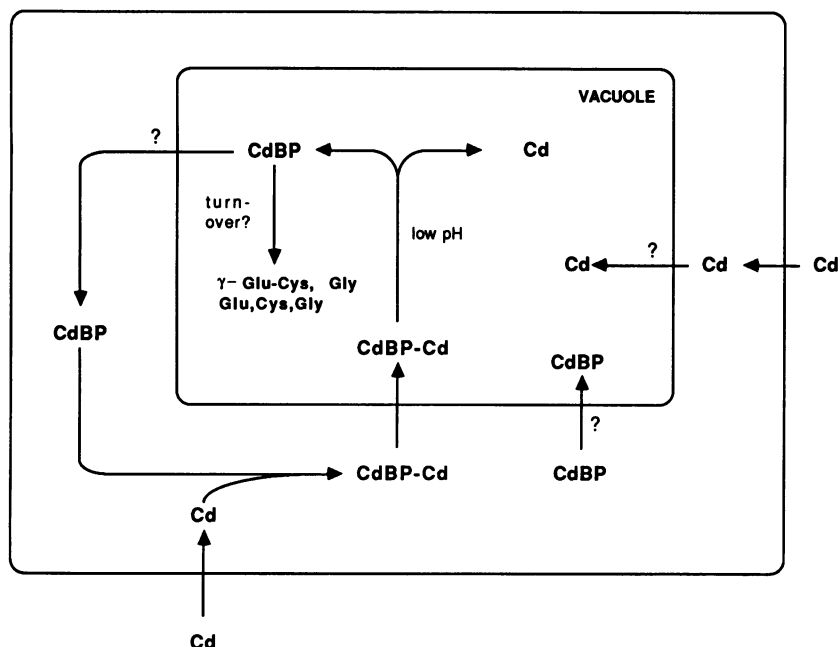


Figure 5. Working model for possible functions of CdBPs (see "Discussion" for explanation).

purified vacuoles. However, because of their relatively low activity in protoplasts, it was not clear if these two enzymes were absent from purified vacuoles or if their activity was below the detection limit (see below for further consideration of this aspect). Microscopic observations, Chl, and NAD-Malate dehydrogenase determinations indicated that the vacuoles were very pure.

Subcellular Localization of CdBPs in Tobacco Leaves

Protoplasts and vacuoles from Cd-exposed tobacco leaves were analyzed by HPLC for the presence of low mol wt thiols (Fig. 4). As expected, GSH as well as CdBPs were found in isolated protoplasts. More interestingly, purified vacuoles also contained CdBPs. CdBPs were not detected in protoplasts and vacuoles from control leaves indicating that stress involved in protoplast isolation does not induce peptide formation (data not shown). Grill *et al.* (8) also found no evidence for the induction of CdBPs after exposure to environmental stress such as heat shock or cold temperature. To compare vacuole samples directly to leaf and protoplast extracts, the amount of sample injected was adjusted to represent equivalent amounts of α -mannosidase activity. With the possible exception of CdBP peak 1, which is only a minor component in the tobacco system, we found similar peak distributions of CdBPs for leaves, protoplasts, and vacuoles. The HPLC-profile of purified vacuoles more closely resembled that of protoplasts than that of leaf extracts. Glutathione was absent from purified vacuoles of tobacco leaves exposed to Cd as described. If a substantial portion of cellular GSH were contained in the vacuole *in vivo* it is unlikely that it would be lost completely during protoplast and vacuole isolation. In a number of studies, low mol wt metabolites thought to be principally contained in the vacuole *in vivo*, were recovered in isolated vacuoles. In this study, protoplast

CdBPs, molecules similar to GSH, were principally recovered in isolated vacuoles (see below).

To quantitatively determine the vacuolar/extravacuolar distribution of Cd and CdBPs we compared the respective amounts of these in protoplasts and vacuoles. Cd was measured by atomic absorption spectroscopy and CdBPs were quantified by integration of the respective HPLC peaks using GSH as a standard for SH. The results from three independent experiments are presented in Table IV. Using α -mannosidase as a vacuolar marker, we found that within the limits of the method used virtually all of the CdBPs were localized in the vacuole. Similarly, virtually all of the Cd was found to be vacuolar. As a control, we added Cd to the bottom layer of a vacuole purification gradient lacking lysed protoplasts and, after centrifugation, the 0/3.9% Ficoll interphase was harvested and analyzed for Cd. No Cd was found under these conditions indicating that Cd associated with the vacuole fraction is not due to unspecific migration of free Cd in the gradient (data not shown). Separation of purified vacuoles into vacuolar sap and membrane fractions (Table V) indicated that CdBPs were associated with the vacuolar sap and not with tonoplast. Since no CdBPs were associated with the tonoplast membrane, the question of whether or not the two membrane markers Cyt *c* oxidase and UDP-glucose ST were absent from vacuoles (see above) becomes less relevant to this study.

In addition to vacuolar CdBPs, small amounts of CdBPs and GSH were also found in the intercellular washing fluid obtained by standard procedures (data not shown). However, since the washing fluid also contained 1 to 2% of the total NAD-malate dehydrogenase activity from leaf extracts, it was not clear if small amounts of CdBPs and GSH occur extracellularly or whether this finding was an artifact due to broken cells. It is interesting to note that Li *et al.* (16) discuss the

possibility of an extracellular pool of NAD-malate dehydrogenase in primary leaves of barley and oat.

DISCUSSION

Addition of 20 μM CdCl_2 to the nutrient solution of hydroponically grown tobacco seedlings induced the synthesis of CdBPs. Under these conditions, the predominant components were γ -(Glu-Cys)₃-Gly and γ -(Glu-Cys)₄-Gly. As reported by Grill *et al.* (8), the number of γ -glutamylcysteinyl repeat units depends on the plant system and on the level of Cd-exposure. We utilized a nonphytotoxic level of Cd to avoid overt stress. Stobart *et al.* (29) reported a 17 or 65% inhibition of Chl biosynthesis in barley leaves exposed to 10 or 100 μM Cd-acetate, respectively. Leaves of tobacco seedlings, grown for 1 week in the presence of 20 μM CdCl_2 , had similar total protein and Chl contents as found in controls (Table I). We also found no significant difference in the GSH content of control and Cd-treated leaves (Fig. 1). Others have reported an initial drop in GSH concentration in response to Cd exposure (6, 8, 19, 25) and as demonstrated by Scheller *et al.* (25), the degree of GSH depletion depends on the Cd concentration and with the lower Cd concentrations, GSH levels can recover over time. Our data indicate that exposure to 20 μM CdCl_2 does not adversely effect growth and viability of tobacco seedlings (Table I).

After examining the vacuolar/extravacuolar distribution of Cd, we found that essentially all Cd was sequestered in the central vacuole (Table IV), thus making this potentially toxic metal unavailable for interaction with metabolically active cellular compartments. Available data on the subcellular localization of Cd are not entirely consistent. This may be due, at least in part, to differences in the methods that have been applied to determine localization. It appears that methods involving transmission electron microscopy and x-ray microanalysis preferentially detect insoluble forms of Cd (10, 20, 27). In *Agrostis* and maize roots exposed to Cd, electron-dense granules containing Cd were found in nuclei, cytoplasm, and vacuoles (20). Heuillet *et al.* (10) detected Cd only in the hypertrophied vacuolar system after exposing the marine alga *Dunaliella bioculata* to high levels of Cd. As stated by Heuillet *et al.* (10) and Rauser and Ackerley (20) possible limitations of this method are leaching of loosely bound Cd during preparation of the tissue for electron microscopy and the possibility that the amount of Cd present in forms other than electron-dense granules might be too low to be detected by x-ray microanalysis. In contrast to electron microscopy studies, methods employing cell fractionation techniques (5, 33) predominantly found Cd associated with the soluble fraction. The limitations of these methods do not allow one to distinguish cytosol and vacuolar sap. Results presented here clearly show vacuolar location of Cd in protoplasts obtained from tobacco leaves exposed to 20 μM Cd. We recently found that most of the Cd and Zn is vacuolar in *Datura innoxia* cell suspension cells exposed to 30 μM Cd or 150 μM Zn (14).

CdBPs have been implicated in detoxification of Cd because of their inducibility by Cd and their high affinity in Cd binding. Additional evidence for involvement of CdBPs in metal tolerance comes from studies utilizing buthionine sulfoximine which is thought to act on the first enzyme of GSH

synthesis, γ -glutamylcysteine synthetase, in plants as in animals. Buthionine sulfoximine is shown to inhibit CdBP synthesis in cultured plant cells (8, 22, 25, 28). Treatment with buthionine sulfoximine generally makes plant cells more susceptible to Cd-toxicity. However, the view of a simple relationship between the occurrence of CdBPs and tolerance to Cd was not supported by a recent study of Delhaize *et al.* (6). Comparing Cd-tolerant and Cd-sensitive cells of *D. innoxia*, they concluded that the synthesis of CdBPs is necessary but not sufficient to express the Cd-tolerant phenotype.

Currently, it seems to be assumed that CdBPs are cytosolic constituents and that detoxification of Cd occurs in the cytosol. Our results obtained by direct comparison of isolated protoplasts and vacuoles from Cd-exposed tobacco leaves clearly show that both Cd and CdBPs are localized in the vacuole. Based on these results we propose that a transport function rather than a simple metal chelation mechanism of CdBPs may be involved in amelioration of Cd-toxicity. This is a mechanism similar to that suggested by Heuillet *et al.* (10) for detoxification of Cd in the marine alga *Dunaliella*. In that study, x-ray microanalysis revealed the presence of Cd in the vacuole in association with organic sulfur. From these results they speculated that Cd may be bound in the cytosol by a 'metallothionein-like' protein followed by transport to and accumulation in the vacuole. However, the nature of the organic sulfur molecule was not identified and no direct evidence for transport was provided.

Figure 5 shows our working model for possible functions of CdBPs in detoxification of Cd. Exposure to the metal induces the synthesis of CdBPs which presumably takes place in the cytoplasm or in chloroplasts since GSH, a proposed substrate for CdBP synthesis (8, 25, 28), appears to be synthesized in these two compartments (13, 15). After their formation, metal-peptide complexes are transported into the vacuole. Due to the generally more acidic pH of the vacuolar sap, Cd-CdBP complexes may dissociate (21). Alternatively, if complexes contain sulfide, which has been shown to increase the stability of metal binding under acidic conditions (23) dissociation may be incomplete. Any free Cd may form complexes with organic acids or amino acids which are present in the vacuole (14). The metal free peptides then may undergo degradation by vacuolar hydrolases or leave the vacuole, so CdBPs could serve as a shuttle mechanism for the translocation of Cd to the vacuole. Alternatively, *de novo* synthesized CdBPs may be the only source of peptide for vacuolar transport of Cd.

At present, we cannot exclude the possibility that Cd and CdBPs may be transported into vacuoles independent of each other. Secondary transport systems coupled to the proton gradient (2, 26) or primary transport systems as suggested for Ca^{2+} transport in tonoplast vesicles from apple fruit (7) may be involved in transport of Cd to the vacuole. However, since CdBPs are very efficient chelators of Cd ($K_{3\text{Cd}} \sim 10^{19}$, see ref. 21) their participation in translocation of Cd to the vacuole could make vacuolar sequestration of Cd more effective, particularly when intracellular Cd levels are high.

In conclusion, we suggest here that amelioration of Cd toxicity in leaves of tobacco seedlings exposed to high levels of Cd does not simply occur by cytoplasmic chelation of Cd

by CdBPs. Rather, toxicity may be avoided by sequestration of Cd in the vacuole. The demonstration of the presence of both, Cd and CdBPs in the vacuole together with the high affinity of CdBPs for Cd binding suggest that CdBPs are involved in translocation of Cd to the vacuole. Further experiments are necessary to directly demonstrate the participation of CdBPs in transport of Cd to the vacuole and to help understand the role of CdBPs in detoxification of Cd.

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