Subcellular localization of cadmium in the root cells of *Allium cepa* by electron energy loss spectroscopy and cytochemistry

DONGHUA LIU[1,2,†] and INGRID KOTTKE[2]

[1]*Department of Biology, College of Chemistry and Life Sciences, Tianjin Normal University, Tianjin 300074, People's Republic of China*

[2]*Eberhard-Karls Universität Tübingen, Botanisches Institut, Spezielle Botanik, Mykologie, Auf der Morgenstelle 1, D-72076 Tübingen, Germany*

[†]*Corresponding author (Fax, 86-22-23358489; Email, donghua@mail.zlnet.com.cn)*

The ultrastructural investigation of the root cells of *Allium cepa* L. exposed to 1 mM and 10 mM cadmium (Cd) for 48 and 72 h was carried out. The results indicated that Cd induced several obvious ultrastructural changes such as increased vacuolation, condensed cytoplasm with increased density of the matrix, reduction of mitochondrial cristae, severe plasmolysis and highly condensed nuclear chromatin. Electron dense granules appeared between the cell wall and plasmalemma. In vacuoles, electron dense granules encircled by the membrane were aggregated and formed into larger precipitates, which increase in number and volume as a consequence of excessive Cd exposure. Data from electron energy loss spectroscopy (EELS) confirmed that these granules contained Cd and showed that significantly higher level of Cd in vacuoles existed in the vacuolar precipitates of meristematic or cortical parenchyma cells of the differentiating and mature roots treated with 1 mM and 10 mM Cd. High levels of Cd were also observed in the crowded electron dense granules of nucleoli. However, no Cd was found in cell walls or in cells of the vascular cylinder. A positive Gomori-Swift reaction showed that small metallic silver grains were abundantly localized in the vesicles, which were distributed in the cytoplasm along the cell wall.

[Liu D and Kottke I 2004 Subcellular localization of cadmium in the root cells of *Allium cepa* by electron energy loss spectroscopy and cytochemistry; *J. Biosci*. **29** 329–335]

1. Introduction

It is well known that Cd is a widespread heavy metal and is released into the environment by power stations, heating systems, metal-working industries, waste incinerators, urban traffic, cement factories and as a by-product of phosphate fertilizers (Sanità and Gabbrielli 1999). Cd has been considered as an extremely significant pollutant affecting all life forms because of its high toxicity and great solubility in soil and water. It has been demonstrated that the level of Cd in the soil appears to be increasing over time (Jones *et al* 1992). Cd accumulation in soil and water now poses a major environmental and human health problem (Schützrndübel *et al* 2001). It was reported that Cd is accumulated by many cereals, potatoes, vegetables and fruits and that humans take up at least 70% of the Cd which originates from plant food (Wagner 1993). Studies on Cd toxicity in plants are well documented. Cd can induce low mitotic index and pycnosis, inhibit cell division and cell proliferation, and has toxic effects on chromosome morphology including c-mitosis, anaphase bridges, chromosome stickiness (Liu *et al* 1992). Cd has toxic effect on nucleoli in the root tip cells of *Allium cepa* L. (Liu *et al* 1995) and alters the synthesis of RNA and impedes ribonuclease acti-

Keywords. *Allium cepa*; Cd, cytochemistry; electron energy loss spectroscopy; root cells

Abbreviations used: CW, Cell wall; C, cytoplasm; CM, cytoplasmic membrane; D, dictyosome; DM, double membrane; EDG, electron dense granules; ER, endoplasmic reticulum; M, mitochondria; NM, nuclear membrane; N, nucleus; Nu, nucleoli; V, vacuoles; Ve, vesicle.

vity in rice (Shah and Dubey 1995). Although the toxicity and tolerance mechanisms of Cd in plants have been widely discussed at the biochemical and cell physiology levels over the last decade (Zenk 1996; Sanità and Gabbrielli 1999; Rauser 1999; Kamnev and Lelie 2000), only a few investigations have been carried out using electron energy loss spectroscopy (EELS) and cytochemical tests to localize intracellular Cd. The objectives of this investigation were to increase our understanding of the effects on root ultrastructure and Cd accumulation sites at subcellular level in the root cells of *A. cepa* grown in different concentrations of Cd solutions, using EELS and cytochemical tests.

2. Materials and methods

2.1 *Plant material and Cd treatments*

Healthy and equal-sized onion cloves were chosen from *A. cepa*. The bulbs were germinated and grown in plastic containers by dipping the base in half Hoagland's nutrient solution (Stephan and Prochazka 1989). The pH of the solution was adjusted to 5·5. The culture room was equipped with supplementary light 15 h light/9 h dark at 20°C. When the roots reached a length of 1 cm, 6 seedlings per treatment were selected, transferred and exposed to different concentrations of Cd solutions for 48 and 72 h, respectively. Cd was applied as cadmium chloride ($CdCl_2 \cdot 2H_2O$) at concentrations of 1 mM and 10 mM in Hoagland solution. The Hoagland nutrient solution was used as the control. The solutions were continuously aerated with an aquarium air pump and changed every 2 days until root preparations were made.

2.2 *Ultrastructure investigations*

2.2a *Chemical fixation*: The treated roots were rinsed in distilled water and cut into segments of 1 mm length at two regions from the root tips – 0–1 mm from root tip meristem and 2–3 mm from the root tip which is considered as mature tissue. The samples were fixed in 3% glutaraldehyde in 0·1 M Hepes buffer (pH 7.2) for 4 h and washed with the same buffer three times. They were post-fixed with 1% osmium tetroxide in the same buffer for 2 h following which the samples were dehydrated in graded acetone and embedded in ERL epoxy resin (Spurr 1969).

Ultrathin sections (80 nm thick) were cut on an ultramicrotome (Ultracut E; Leica, Germany) by a diamond knife. The sections were mounted on copper grids, and stained with 1% uranyl acetate for 1 h and lead citrate for 15 min. Observations and photography were accomplished on transmission electron microscope (TEM 902; Zeiss, Germany).

2.3 *EELS analysis*

Sections of 40 nm thickness from fixed material were cut and mounted on 600 mesh copper grids without formvar coating. Identification and localization of Cd were carried out using EELS connected to the TEM 902 (Zeiss, type Castaing-Ottensemyer, serial energy-loss spectrum). The energy loss spectra of selected areas was 380–550 eV for Cd-$M_{4,5}$ (E = 403). An acceleration voltage of 80 kV and 50,000–85,000 time magnifications was operated corresponding to an area diameter of 0·3 μm and 0·18 μm, respectively. Objective aperture of 30 μm and a spectrometer entrance aperture of 100 μm were used.

The beam current was 50 μA at an energy-selecting slit width of 2 eV, with a step size of 0·5 eV, and a dwell time of 0·5 s per channel.

2.4 *Cytochemical tests*

2.4a *Gomori-Swift reaction to detect cysteine-rich protein*: Sections of 100 nm thickness from fixed material were cut and mounted on gold grids.

Solution A containing 5 ml of 5% silver nitrate and 100 ml of 3% hexamethylenetetramine and solution B consisting of 10 ml 1·44% boric acid and 100 ml 1·9% borax were prepared. The final stain was obtained by mixing 25 ml of A, 5 ml of B and 25 ml of distilled water. The grids were floated in the silver methenamine solution for 90 min at 45°C in the dark, and were then washed four times for 2 min. The grids were then floated on 10% sodium thiosulphate solution for 1 h at room temperature to dissolve metallic silver and rinsed in deionized water four times for 2 min. The sections were continuously stained with uranyl acetate and lead citrate (Swift 1968).

Controls were carried out to block S-H group and S-S group by the reduction of disulfide bonds in benzylmercaptan followed by alkylation of S-H groups in iodacetate/boric acid. The procedures were described by Swift (1969).

3. Results

3.1 *Ultrastructure observation*

The root cells of control plants had a typical ultrastructure. Large amounts of ribosomes, dictyosomes and endoplasmic reticulum (ER) were present in the dense cytoplasm. The mitochondria were oval with well-developed cristae (figure 1a). Several small vacuoles in the meristem or one large vacuole were found in mature root cells.

Root cells exposed to concentrations of 1 mM and 10 mM of Cd exhibited pronounced changes. In contrast to the control, the increased vacuolation was the first visible effect of metal toxicity on the meristematic cells treated with

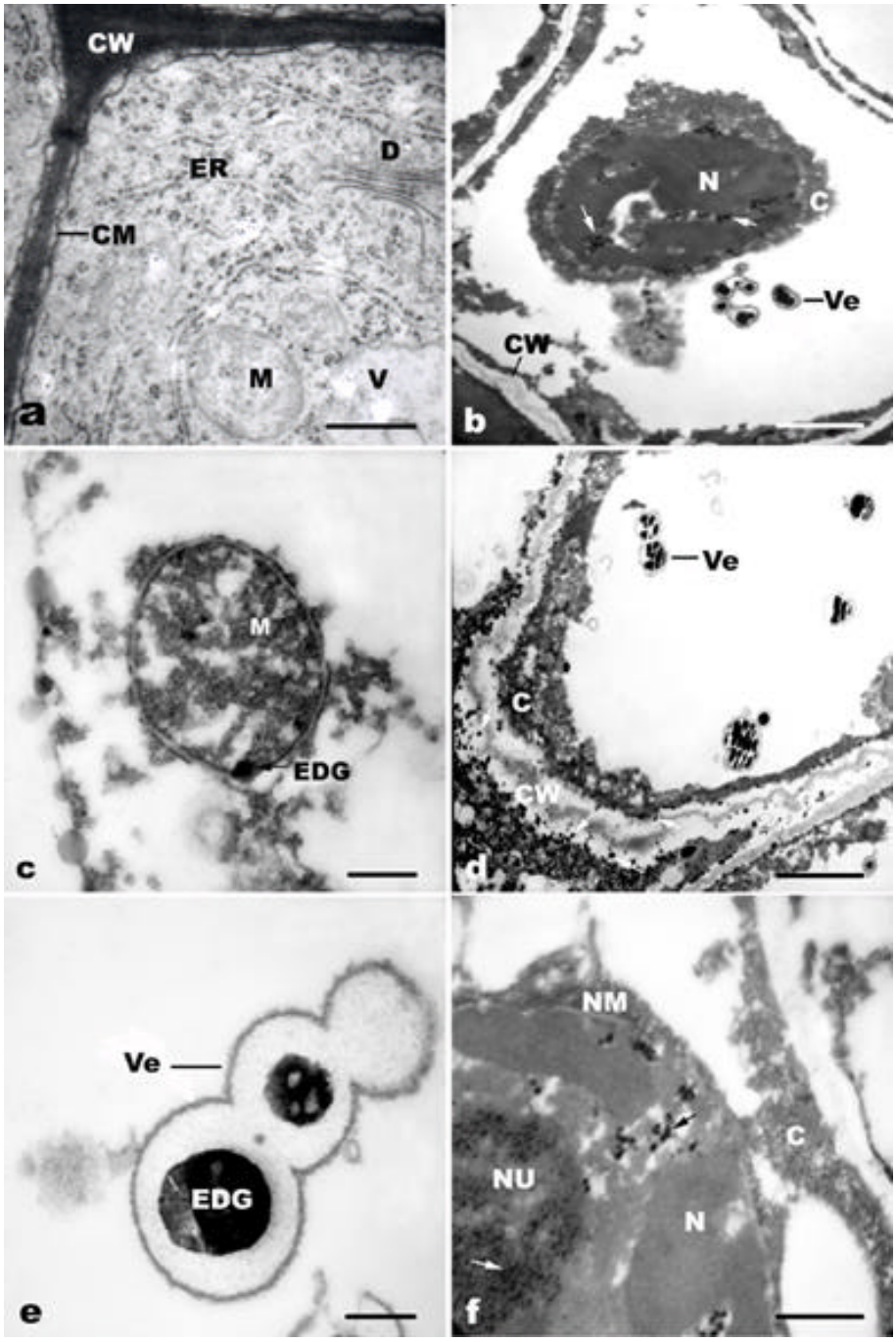

Figure 1. TEM micrographs showing toxic effects of Cd on ultrastructure of the root tip cells of *A. cepa*. **(a)** Control cells showing well developed root tip cells. **(b–f)** The ultrastructural changes of root tip cells treated with 1 mM–10 mM Cd for 48 and 72 h. **(b)** A highly condensed cytoplasm lining the cell wall and around the nucleus in most of cortical cells, and electron dense granules with membrane distributed in vacuoles (1 mM Cd, 48 h). **(c)** Reduction of mitochondrial cristae and accumulation of some electron dense granules (10 mM Cd, 48 h). **(d)** The amount of electron dense granules in between cell wall and plasmalemma and plasmolysis of cortical cells (1 mM Cd, 72 h). **(e)** Treated cells at high magnification showing electron dense granules enclosed within membranous structures and distributed in vesicles (10 mM Cd, 72 h). **(f)** Electron dense granules scattered in nucleoli (10 mM Cd, 48 h). Bar = 0.2 μm **(c)**; Bar = 0.25 μm **(d**, **e)**; Bar = 0.5 μm **(a**, **f)**; Bar = 1 μm **(b)**. Arrows showing electron dense granules.

1 mM Cd. Condensed cytoplasm with increased density of the matrix was observed in most of cortical cells, with only a little cytoplasm lining the cell wall and around the nucleus (1 mM Cd, 48 h; figure 1b). Reduction in the number of mitochondrial cristae was often found (figure 1c). Severe plasmolysis occurred in cortical cells (figure 1b, d). The nuclear chromatin appeared highly condensed (figure 1b). These structural alternations led to death of most cells treated with 10 mM Cd for 72 h.

The amounts of electron dense granules were also observed between the cell wall and plasmalemma (figure 1d). In vacuoles, electron dense granules were aggregated and formed into larger precipitates with circular or amorphic shape, which were encircled by the membrane (figure 1b, d, e). These appeared to increase in number and volume in vacuoles and cytoplasm as a consequence of excessive Cd-exposure from 1 mM Cd to 10 mM Cd.

Electron dense granules were also found to be scattered in nucleoli (figure 1b, f), trace amounts were noticed in the mitochondrial matrix (figure 1c) as well as in cell walls of meristematic and cortical parenchyma cells after Cd stress for 48 and 72 h.

3.2 *Cytochemistry (Gomori-Swift reaction)*

Histochemical test by Gomori-Swift reaction is highly sensitive and allows the detection of cysteine-rich proteins which might play an important role in element detoxification. In the present investigation, control root cells did not react to the Gomori-Swift treatment (figure 2a). Gomori-Swift reaction exhibited the presence of cysteine-rich proteins in different regions of Cd-treated cells. Small metallic silver grains as a result of positive reaction were abundantly localized in the vesicles, which were distributed in cytoplasm along the cell walls (figure 2b). Usually, the vesicles with metallic silver grains are encircled by the double membrane (figure 2c) in cytoplasm. Trace amounts of silver grains were found in bigger precipitates localized in the vacuoles or in the cell walls of cortical cells. However, in the nuclei, Gomori-Swift reaction was not obvious even though such metallic silver grains were present near the nuclear membrane (figure 2d).

3.3 *Electron energy loss spectroscopic analysis*

EEL-spectra of the Cd-$M_{4,5}$ edge gave clear indications that Cd was localized in the electron dense granules deposited in various cell regions at 1 and 10 mM Cd stress for 48 h or 72 h. These electron dense granules appeared bright when observed at 250 eV. A significantly higher level of Cd in vacuoles was found in the vacuolar precipitates of meristematic or cortical parenchyma cells of the differentiating and mature roots treated with 1 mM and 10 mM Cd, where the typical Cd peak was fully confirmed by EELS (figure 3). Low levels of Cd was detected in some vesicles lining plasmalemma or mitochondria matrix when compared with vacuolar precipitates. A high level of Cd was also noticed in the crowded electron dense granules of nucleoli. However, the nucleoplasm contained dispersed electron dense granules indicating that only trace amounts of Cd existed in it. No Cd was found in cell walls or in cells of the vascular cylinder.

4. Discussion

Identification of sites of localization of heavy metals at the subcellular level in cell organelles, cytoplasm or cell walls, would help to clarify the process involved in their uptake, transport and deposition or detoxification in plant cells. EELS is a good method with many advantages: EELS can present a better energetic resolution and a higher sensitivity than X-ray microanalysis (Castaing 1987) and EELS can determine the oxidation states of ionized elements (Mansot *et al* 1994). EELS is more sensitive than both energy-dispersive X-ray microanalysis (EDX) and secondary-ion mass-spectrometry (SIMS) (Huxham *et al* 1999).

4.1 *Effects of Cd on root cell structure of* A. cepa

The results from the present investigation showed that in *A. cepa* the Cd toxicity is realized at two levels: (i) low Cd concentration can induce morphological alterations and lead to increased vacuolation and formation of small cytoplasmic vesicles, which is a common feature induced by heavy metals at low concentration (Smith 1983; Nassiri *et al* 1997a, b; Sresty *et al* 1999). Sanità and Gabbrielli (1999) indicated that vacuolar compartmentalization plays a very significant role in Cd detoxification and tolerance, which prevents the circulation of free Cd ions in the cytosol and forces them into a limited area. Recent investigations have shown that low Cd concentrations do not affect plant growth (Barceló and Poschenrieder 1999). Our earlier study showed that Cd stimulated root length at lower concentrations (10^{-5}–10^{-6} M) significantly ($P < 0{\cdot}005$) (Jiang *et al* 2001). The changes in morphology mentioned above do not affect plant growth obviously. (ii) At high concentration of Cd (1 mM and 10 mM), the membrane system of the cell is damaged, leading to condensed cytoplasm, reduction in the number of mitochondrial cristae, severe plasmolysis and decrease in number of ribosomes. These structural alterations ultimately lead to death of most cells and obviously affect plant root growth.

4.2 *Localization of Cd in the root tip cells of* A. cepa

EELS can accurately show the route of transport and storage sites of Cd at ultrastructural level. Data from EELS generated during this study confirm that Cd exists in the electron dense granules. Vacuoles are main storage sites of Cd in the root tip cells of *A. cepa*, which support the findings of Nassiri *et al* (1997a, b). The electron dense granules are a morphological index of Cd in the cells and were seen enclosed in vesicles. These were formed on the cell wall, enclosed by membrane and were then transported from cytoplasm into vacuoles. These granules containing Cd can be thought to be a mechanism for detoxification of Cd. Nucleoli were found to be another storage site of Cd in the cells according to the results in the present investigation.

It was reported that cell wall can play a role in metal tolerance as its volume may be high compared to the cytosol and vacuole (Neumann *et al* 1997). According to the reports on subcellular localization of Cd in the cell walls, the results are quite conflicting. Some studies showed that Cd is mainly accumulated in cell walls when its content is high (Lindsey and Lineberger 1981; Khan *et al* 1984; Küpper *et al* 2000) while others indicated that Cd mainly found within vacuoles and nuclei (Rauser and Ackerley 1987). In the present work using EELS analytical tech-

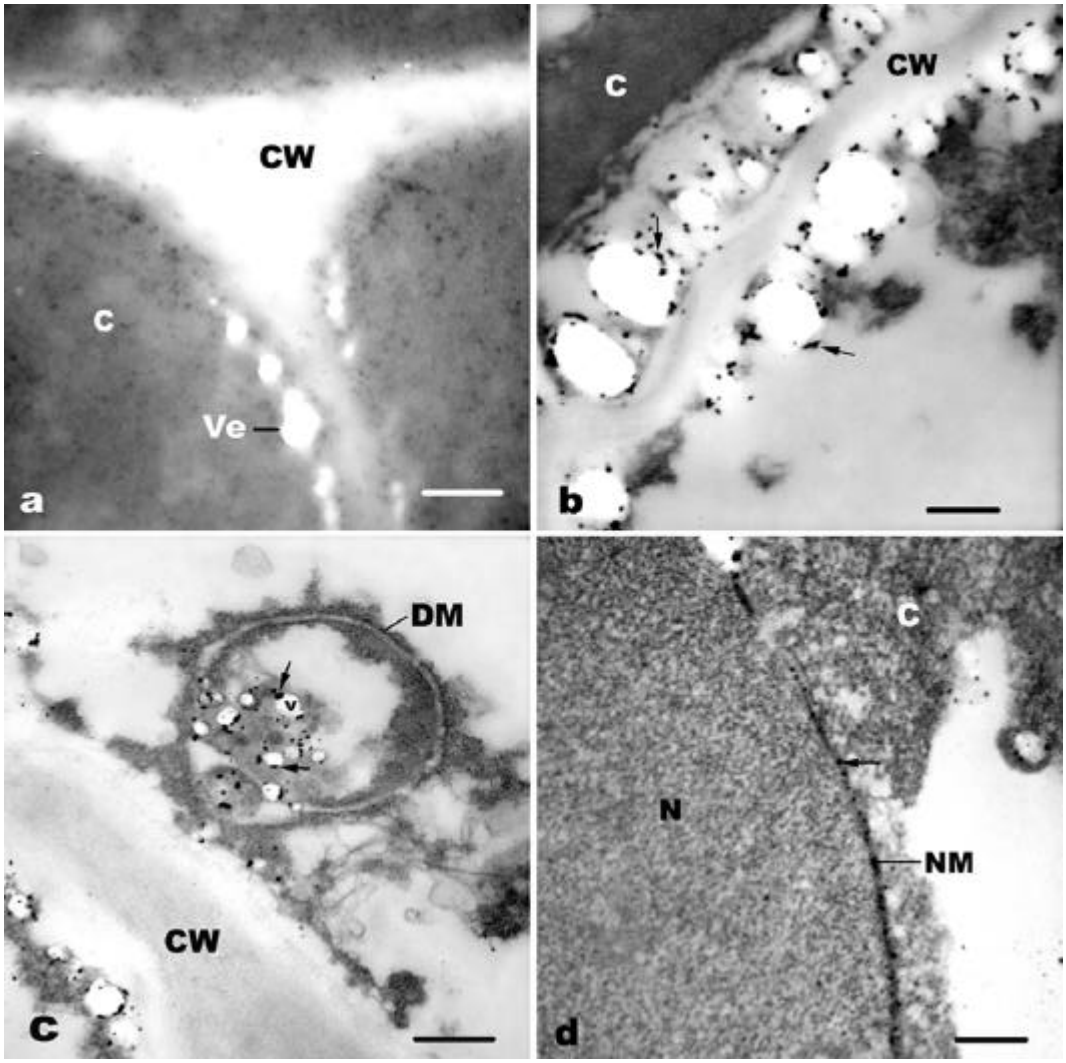

Figure 2. Cytochemical test of the root tip cells of *A. cepa*. **(a)** Without showing Gomori-Swift reaction in the cell of control. **(b)** Metallic silver grains with Gomori-Swift positive reaction localized in the cytoplasmic vesicles along the cell walls (10 mM Cd, 48 h). **(c)** Small vesicles with metallic silver grains encircled by double membrane in the cytoplasm (10 mM Cd, 72 h). **(d)** Metallic silver grains with Gomori-Swift reaction localized in nuclear membrane (1 mM Cd, 72 h). Bar = 0.1 μm **(d)**; Bar = 0.2 μm **(a, b, c)**. Arrows showing electron dense granules.

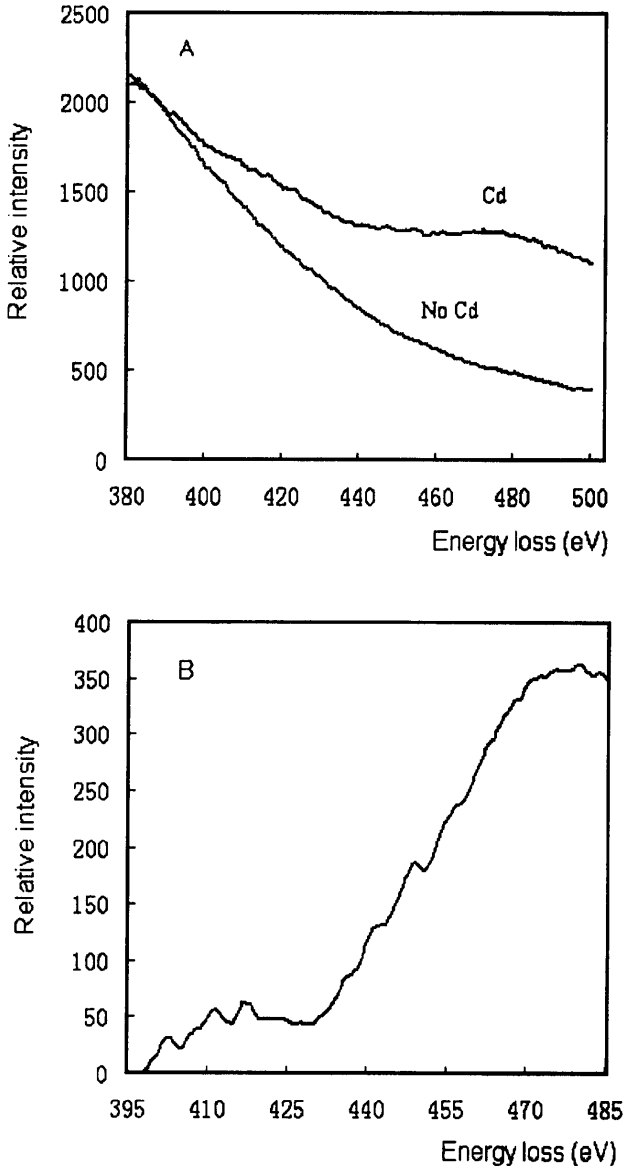

Figure 3. EELS. **(A)** Cadmium spectrum obtained from vacuolar precipitates of cortical parenchyma cells in the roots of *A. cepa* treated with 1 mM Cd. **(B)** 'Stripped' spectrum obtained from vacuolar precipitates containing cadmium.

nology, no Cd was detected in the cell wall. The conflicting results can be due to differences between the plant species and their capacities to accumulate and sequester toxic metals, as well as to differences in the experimental methods and conditions (Turnau *et al* 1996). However, Gomori-Swift reaction showed that trace amounts of silver grains were found in the cell walls of cortical cells, indirectly suggesting that Cd may be distributed in the cell walls due to existence of cysteine-rich proteins (phytochelatins, PCs) in the cell walls.

The observation by TEM and cytochemical reaction confirmed that the vesicles and some mitochondria contain cysteine-rich proteins. However, the bigger precipitates containing Cd in the vacuoles only exhibited a weak Gomori-Swift positive reaction, suggesting that after Cd ions enter the cytosol, sulphur metabolism is promptly activated resulting in the production of cysteine-rich proteins, phytochelatins (Sanità and Gabbrielli 1999). Cd ions bind phytochelatins and form stable PC/Cd complexes. These are transported across the tonoplast and sequestered in the plant vacuoles (Saxena *et al* 1999). After the complexes enter the vacuole, Cd is released from the phytochelatins, which are then returned to the cytoplasm (Greger 1999). Cd can be complexed by vacuolar organic acids (Krotz *et al* 1989). These may explain why Gomori-Swift shows a weak positive reaction in the vacuoles.

Acknowledgements

The work, undertaken in Botanisches Institut, Spezielle Botanik, Eberhard-Karls Universität Tübingen, Germany, was supported by grant of a DAAD/KC Wong Fellowship to D H Liu and by the National Natural Science Foundation of China.

References

Barceló J and Poschenrieder C 1999 Structural and ultrastructural changes in heavy metal exposed plants; in *Heavy metal stress in plants* (eds) M N V Prasad and J Hagemeyer (Berlin, Heidelberg: Spinger-Verlag) pp 183–205

Castaing R 1987 Progrès récents en microanalyse des solides; *Analusis* **15** 517–527

Greger M 1999 Metal availability and bioconcentration in plants; in *Heavy metal stress in plants* (eds) M N V Prasad and J Hagemeyer (Berlin, Heidelberg: Spinger-Verlag) pp 1–27

Huxham I M, Jarvis M C, Shakespeare L, Dover C J, Knox J P and Seymour G B 1999 Electron energy loss spectroscopic imaging of calcium and nitrogen in the cell walls of apple fruits; *Planta* **208** 438–443

Jiang W S, Liu D H and Hou W Q 2001 Hyperaccumulation of cadmium by roots, bulbs and shoots of *Allium sativum* L; *Bioresource Tech*. **76** 9–13

Jones K C, Jackson A and Johnston A E 1992 Evidence for an increase in the Cd content of herbage since 1860's; *Eviron. Sci. Technol*. **26** 834–836

Kamnev A A and Lelie van der D 2000 Chemical and biological parameters as tools to evaluate and improve heavy metal phytoremediation; *Biosci. Rep*. **20** 239–258

Khan D H, Duckett J G, Frankland B and Kirkham J B 1984 An X-ray microanalytical study of the distribution of cadmium in roots of *Zea mays* L; *J. Plant Physiol*. **115** 19–28

Krotz R M, Evangelou B P and Wagner G J 1989 Relationships between cadmium, zinc, Cd-binding peptide and organic acid in tobacco suspension cells; *Plant Physiol*. **91** 780–787

Küpper H, Lombi E, Zhao F-J and McGrath S P 2000 Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator; *Planta* **212** 75–84

Lindsey P A and Lineberger R D 1981 Toxicity, cadmium accumulation and ultrastructural alterations induced by exposure of Phaseolus seedlings to cadmium; *Hortscience* **16** 434

Liu D H, Jiang W S and Li M X 1992 Effects of Cd^{2+} on root growth and cell division of *Allium cepa*; *Acta Sci. Circumstantiae* **12** 439–446

Liu D, Jiang W, Wang W and Zhai L 1995 Evaluation of metal ion toxicity on root tip cells by the Allium test; *Isr. J. Plant Sci.* **43** 125–133

Mansot J L, Leone P, Euzen P and Palvadeau P 1994 Valence of manganes, in a new oxybromide compound, determined by means of electron energy loss spectroscopy; *Microsc. Microanl. Microstruct.* **5** 79–90

Nassiri Y, Wéry Mansot J L and Ginsburger-Vogel T 1997a Cadmium bioaccumulation in *Tetraselmis suecica*: an electron energy loss spectroscopy (EELS) study; *Arch. Environ. Contam. Toxicol.* **33** 156–161

Nassiri Y, Mansot J L, Wéry J, Ginsburger-Vogel T and Anuard C J 1997b Ultrastructureal and electron energy loss spectroscopy studies of sequestration mechanisms of Cd and Cu in the marine diaton *Skeletonema costatum*; *Arch. Environ. Contam. Toxicol.* **33** 147–155

Neumann D, zur Nieden U, Schwieger W, Leopold I and Lichtenberger O 1997 Heavy metal tolerance of *Minuartia erna*; *J. Plant Physiol.* **151** 101–108

Rauser W E 1999 Structure and function of metal chelators produced by plants; *Cell Biochem. Biophy.* **31** 19–48

Rauser W E and Ackerley C A 1987 Localization of cadmium in granules within differentiating and mature root cells; *Can. J. Bot.* **65** 643–646

Sanità L di Toppi and Gabbrielli R 1999 Response to cadmium in higher plants; *Environ. Exp. Bot.* **41** 105–130

Saxena P K, KrishnaRaj S, Dan T, Perras M R and Vettakkorumakankav N N 1999 Phytoremediation of heavy metal contaminated and polluted soils; in *Heavy metal stress in plants* (eds) M N V Prasad and J Hagemeyer (Berlin, Heidelberg: Spinger-Verlag) pp 305–329

Schützrndübel A, Schwanz P, Teichmann T, Gross K, Langenfed-Heyser R, Godbold D L and Polle A 2001 Cadmium-induced changes in antioxidative system, hydrogen peroxide content and differentiation in scots pine roots; *Plant Physiol.* **127** 887–898

Shah K and Dubey R S 1995 Effect of cadmium on RNA level as well as activity and molecular forms of ribonuclease in growing rice seedlings; *Plant Physiol. Biochem.* **33** 577–584

Smith M A 1983 The effect of heavy metals on the fine structure of *Skeletonema costatum* (Bacillarophyta); *Protoplasma* **116** 14–23

Sresty T V and Madhava Rao K V 1999 Ultrastructural alterations in response to zinc and nickel stress in the root cells of pigeonpea; *Environ. Exp. Bot.* **41** 3–13

Spurr A R 1969 A low-viscosity epoxy resin embedding medium for electron microscopy; *J. Ultrastruct. Res.* **26** 31–43

Stephan U W and Prochazka Z 1989 Physiological disorders of the nicotianamine-auxotroph tomato mutant *chloronerva* at different levels of iron nutrition. I. Growth characteristics and physiological abnormalities as related to iron and nicotianamine supply; *Acta Bot. Nerrl.* **38** 147–153

Swift J A 1968 The electron histochemistry of cysteine-containing proteins in thin transverse sections of human hair; *J. Micros.* **88** 449–460

Swift J A 1969 The electron histochemical demonstration of cystine-containing proteins in the guinea pig hair follicle; *Histochemie* **19** 88–98

Turnau K, Kottke I and Dexheimer J 1996 Toxic element filtering in *Rhizopogon roseolus/Pinus sylvestris* mycorrhizas collected from calamine dumps; *Mycol. Res.* **100** 16–22

Wagner G J 1993 Accumulation of cadmium in crop plants and its consequences to human health; *Adv. Agron.* **51** 173–212

Zenk M H 1996 Heavy metal detoxification in higher plants – a review; *Gene* **179** 21–30

MS received 15 November 2003; accepted 10 August 2004

Corresponding editor: DEEPAK PENTAL