

# Subcellular localization of Cd in the root cells of *Allium sativum* by electron energy loss spectroscopy

DONGHUA LIU<sup>1,2,†</sup> and INGRID KOTTKE<sup>2</sup>

<sup>1</sup>Department of Biology, College of Chemistry and Life Sciences, Tianjin Normal University, Tianjin 300074, People's Republic of China

<sup>2</sup>Eberhard-Karls-Universität Tübingen, Botanisches Institut, Spezielle Botanik, Mykologie, Auf der Morgenstelle 1, D-72076 Tübingen, Germany

<sup>†</sup>Corresponding author (Fax, 86-22-23358489; Email, donghua@mail.zlnet.com.cn)

The ultrastructural investigation of the root cells of *Allium sativum* L. exposed to three different concentrations of Cd (100 µM, 1 mM and 10 mM) for 9 days was carried out. The results showed that Cd induced several significant ultrastructural changes – high vacuolization in cytoplasm, deposition of electron-dense material in vacuoles and nucleoli and increment of disintegrated organelles. Data from electron energy loss spectroscopy (EELS) revealed that Cd was localized in the electron-dense precipitates in the root cells treated with 10 mM Cd. High amounts of Cd were mainly accumulated in the vacuoles and nucleoli of cortical cells in differentiating and mature root tissues. The mechanisms of detoxification and tolerance of Cd are briefly explained.

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## 1. Introduction

Cadmium is a particularly dangerous pollutant due to its high toxicity and great solubility in water (Lockwood 1976). Considerable importance has been attached to the problems associated with Cd pollution, because modern industrial activities and agricultural practices increase Cd levels in the soil. The possibility of using specific plants which hyperaccumulate metals to selectively remove and recycle excessive soil metals was first introduced by Chaney (1983). Phytoremediation has been also defined by Salt *et al* (1996) as an emerging technology for environmental clean-up. The importance for phytoremediation is that specially selected and engineered metal-accumulating plants are able to transfer the heavy metal efficiently and rapidly from soil via their root system to the shoot. Thereafter the plants are harvested and disposed off. The investigation indicated that garlic (*Allium sativum* L.) can have considerable ability to accumulate substantial amounts of cadmium (Jiang *et al* 2001).

It has been reported that heavy metal tolerance in higher plants is the result of different processes which prevent excess and toxic heavy metal concentrations in the cytoplasm and organelles. These processes involve alteration of plasma membrane structures, induction of the synthesis of heat shock proteins, chelation with polyhydroxy phenolic compounds and so on (Neumann *et al* 1995, 1997; Bringezu *et al* 1999). Although the toxic and tolerant mechanisms of Cd in plants were widely discussed over the last decade (Sanità and Gabbrielli 1999; Rauser 1999; Zenk 1996; Kamnev and van der Lelie 2000), only a few investigations have been carried out using electron energy loss spectroscopy (EELS). Knowledge of the subcellular localization and identification of cadmium can provide essential information on metal toxicity and bioaccumulation mechanisms. EELS is a good method for cadmium detection and quantification in biological specimens (Nassiri *et al* 1997a,b), and is more sensitive than both energy-dispersive X-ray microanalysis (EDX) and secondary-ion mass-spectrometry (SIMS) (Huxham *et al* 1999).

**Keywords.** *Allium sativum*; EELS; Cd; Gomori-Swift reaction

The objectives of this investigation were to increase our understanding of the ultrastructural damages and storage sites of Cd in the root cells of *A. sativum* grown in the different concentrations of Cd solutions at subcellular level, using the technique of EELS.

## 2. Materials and methods

### 2.1 Plant material, growth conditions and metal treatments

Healthy and equal-sized garlic cloves were chosen from *A. sativum*. The bulbs had not started the formation of green leaves or root growth. Before commencing the experiment, the dry scales of the bulbs were removed. The bulbs were germinated, and grown in 3 containers with 2 L Hoagland's nutrient solution (Stephan and Prochazka 1989) in a greenhouse equipped with supplementary 14 h light/9 h dark at 18°C~20°C diurnal cycle. The composition of Hoagland's solution was 5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 5 mM KNO<sub>3</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 50 µM H<sub>3</sub>BO<sub>3</sub>, 1 mM MgSO<sub>4</sub>, 4.5 µM MnCl<sub>2</sub>, 3.8 µM ZnSO<sub>4</sub>, 0.3 µM CuSO<sub>4</sub> and 0.1 mM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> and 10 µM FeEDTA. The pH was adjusted 5.5.

Grown in the half-concentrated nutrient solutions for two days, 10 seedlings for each treated-group were selected, transplanted and grown hydroponically in one container (4 cm tall, 30 × 20 cm) with the 2 L Hoagland's nutrient solution. Different concentrations of aerated Cd solution ranging from 10 mM, 1 mM and 100 µM Cd, respectively were added. Cd was provided as cadmium chloride (CdCl<sub>2</sub>·2.5 H<sub>2</sub>O). The Cd solutions were prepared with deionized water. The test solutions were changed every 3 days. Deionized water also was used as the control experiment.

### 2.2 Transmission electron microscopy investigation

The roots were harvested after 9 days of culture. The terminal root 0–1 mm and 2–3 mm from each root of control and treated groups were cut and fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 4 h and were thoroughly washed with the same buffer for three times. This was followed by postfixation with 2% osmium tetroxide in the same buffer for 2 h. They were dehydrated in a series of acetone, and embedded in ERL resin. For ultrastructure observations, ultrathin sections of 80 nm thickness were cut on an ultramicrotome (Ultracut E, Leica, Germany) with a diamond knife, and were picked on copper grids. The sections were stained with 1% uranyl acetate for 1 h and lead citrate for 15 min. They were examined on and photographed with transmission electron microscopy (TEM 902, Zeiss, Germany).

### 2.3 Electron energy loss analysis

For the EELS viewing, the sections were cut at 40 nm thickness and collected on 600 mesh Ni grids not formvar coated. Cd content was measured using EELS spectra of a TEM 902 (Zeiss, type Castaing-Ottensmeyer, serial energy-loss spectrum) operating at 80 kV. And 50,000~85,000 time magnifications was operated corresponding to an area diameter for analysis 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 energy loss range for Cd was  $E = 380\text{--}540$  eV, to detect the Cd-M<sub>4,5</sub> edge at 403.7 eV. For detection of P, S, O and Ca, the EEL-spectra of selected areas were: P-L<sub>2,3</sub> edge at 132.2 eV, S-L<sub>2,3</sub> edge at 164.6 eV, O-K edge at 532 eV and Ca-L<sub>2,3</sub> at 346.4 eV, respectively. 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 was 0.5 s per channel.

## 3. Results

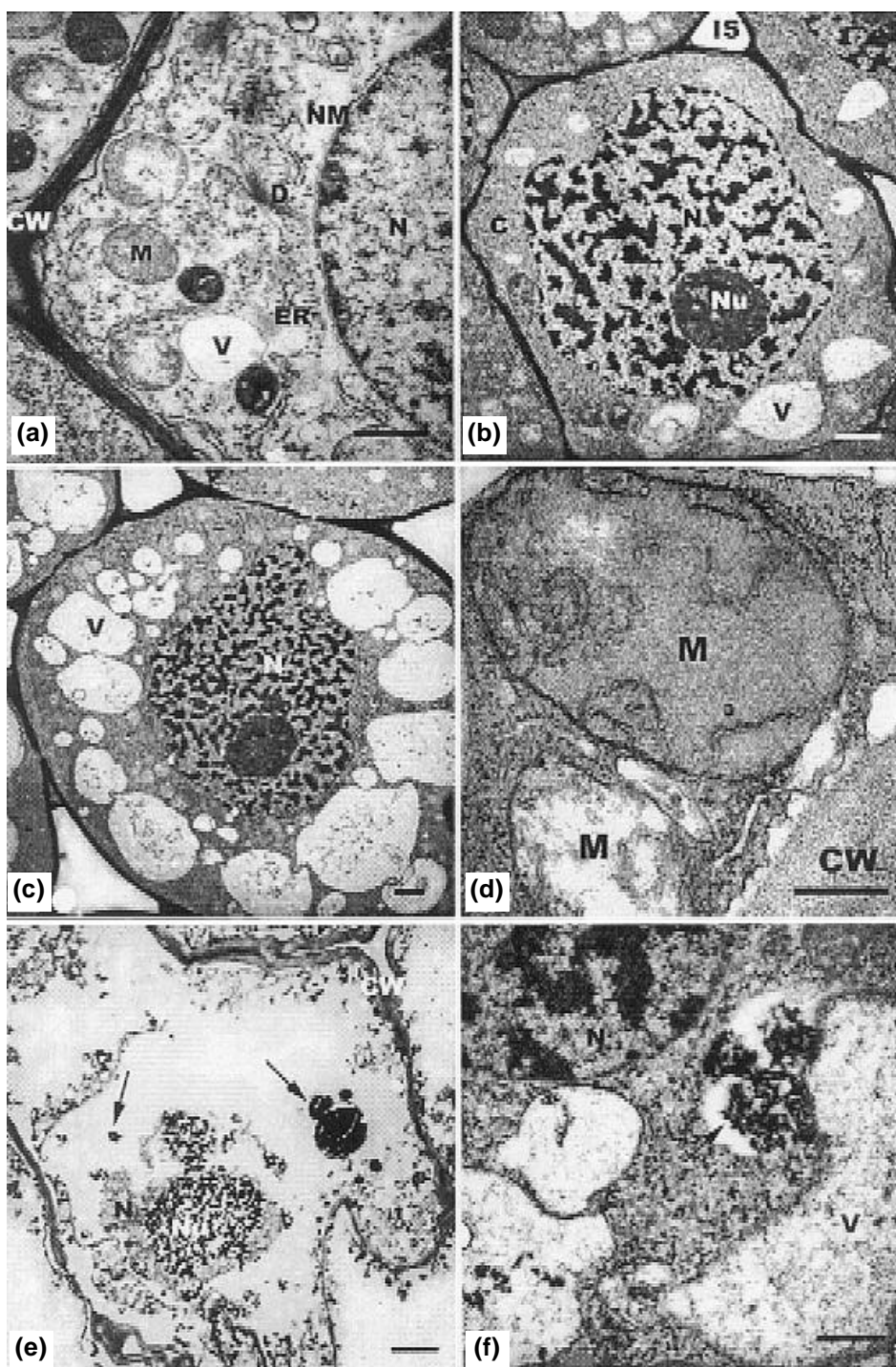
### 3.1 Effects of Cd on ultrastructures

Root tip cells of garlic control plants have a typical ultrastructure. In control cells, usually highly condensed cytoplasm contains numerous organelles and a large nucleus. Rich ribosomes are distributed in cytoplasm or located on the surface of endoplasmic reticulum (figure 1a). Some small vacuoles are found in meristematic cells, whereas a large vacuoles or several vacuoles are exhibited in mature parenchyma cell of the root tip (figure 1b).

The root cells exposed to different concentrations of Cd exhibited various ultrastructural changes when compared to control root cells. At 100 µM Cd, advanced vacuolation was mainly the toxic symptoms which appeared in the meristem and cortical cells of root tips (figure 1c). This was prominent in cortical parenchyma cells than in staler parenchyma cells of the mature root tissues.

From concentrations 1 to 10 mM Cd, the structures of cytoplasm and organelles, both in cortex and stelar parenchyma tissues, were, in varying degrees, damaged. A cirstae reduction in number was observed in mitochondria (figure 1d). The multivesiculate bodies were more exhibited in parenchyma cells (1 mM Cd).

Nucleus disintegration was accompanied the nucleoli containing rich electron-dense granules (figure 1e). Damaged membrane systems and serious plasmolysis with separations of the plasma membrane from the cell wall were noted in most root cells of the mature root tissues exposed to 10 mM Cd. The structural damaged of the cells resulted in death of most of the cells, which gradually decreased from cortical cells to staler cells.



**Figure 1.** TEM micrographs showing toxic effects of cadmium on ultrastructure in the root tip cells of *Allium sativum*. (a–b) Control cells showing well developed root tip cells. (c–f) The ultrastructural changes of root tip cells treated with 100  $\mu\text{M}$  – 10 mM Cd for 9 days. (c) Showing increase in vacuolation (100  $\mu\text{M}$  Cd). (d) Showing reduction of mitochondria (1 mM Cd). (e) Showing large precipitates encircled by membranes in the vacuoles and nucleoli containing electron-dense granules (10 mM Cd). (f) Showing a few electron-dense granules distributed in vesicles in the cytoplasm (1 mM Cd). Bar = 0.25  $\mu\text{m}$  (d); Bar = 0.5  $\mu\text{m}$  (a, f); Bar = 1  $\mu\text{m}$  (b, c, e). Arrows point to electron-dense granules. CW, Cell wall; C, cytoplasm; D, dictyosome; ER, endoplasmic reticulum; IS, intercellular space; M, mitochondria; N, nucleus; NM, nuclear membrane; Nu, nucleoli; V, vacuoles.

A few electron-dense granules precipitated in large vacuoles or in small vesicles in cytoplasm of the cortical parenchyma cells which were treated with 100  $\mu$ M to 1 mM Cd (figure 1f). The precipitation increased with increasing concentrations of Cd. Cd was identified in these electron dense granules by EELS. At 10 mM Cd, the abundant electron dense granules containing Cd formed into bigger precipitates which were encircled by membrane in the vacuoles (figure 1e). Small amounts were scattered in the nucleus (figure 1e) and in the cytoplasm (figure 1f) of the cortical parenchyma cells.

### 3.2 Electron energy loss spectroscopic analysis

EEL spectra of the Cd-M 4-5 edge clearly indicated that cadmium was present in electron-dense granules deposited in various cell regions (under 10 mM Cd stress for 9 days). These electron-dense granules appeared bright when observed at 250 eV. The significantly higher level of Cd was found in vacuoles of meristematic or parenchyma cells – in the differentiating and mature roots. These granules containing Cd were accumulated and deposited to be formed into bigger precipitates, where the typical Cd peak was confirmed by EELS, as shown in figure 2A,B. Within these precipitates the following elements were detected: P, C, O and Ca (figure 3A–C). In addition, in vesicular precipitates from cytoplasm, the level of Cd was usually lower in comparison with vacuolar precipitates, indicating that this phenomenon resulted from only a few electron-dense granules containing Cd being spread in them. The lack of Cd signals from cell walls could be due to their low concentrations.

High content of Cd (figure 2C,D) was found by EELS in nucleoli of some cortical parenchyma cells where rich electron-dense material existed. The electron-dense granules with Cd in nucleoli always accompanied amounts of C and O, while only a little Ca and P were detected (figure 3D,E). In the cells treated with 100  $\mu$ M and 1 mM Cd, Cd was not found.

## 4. Discussion

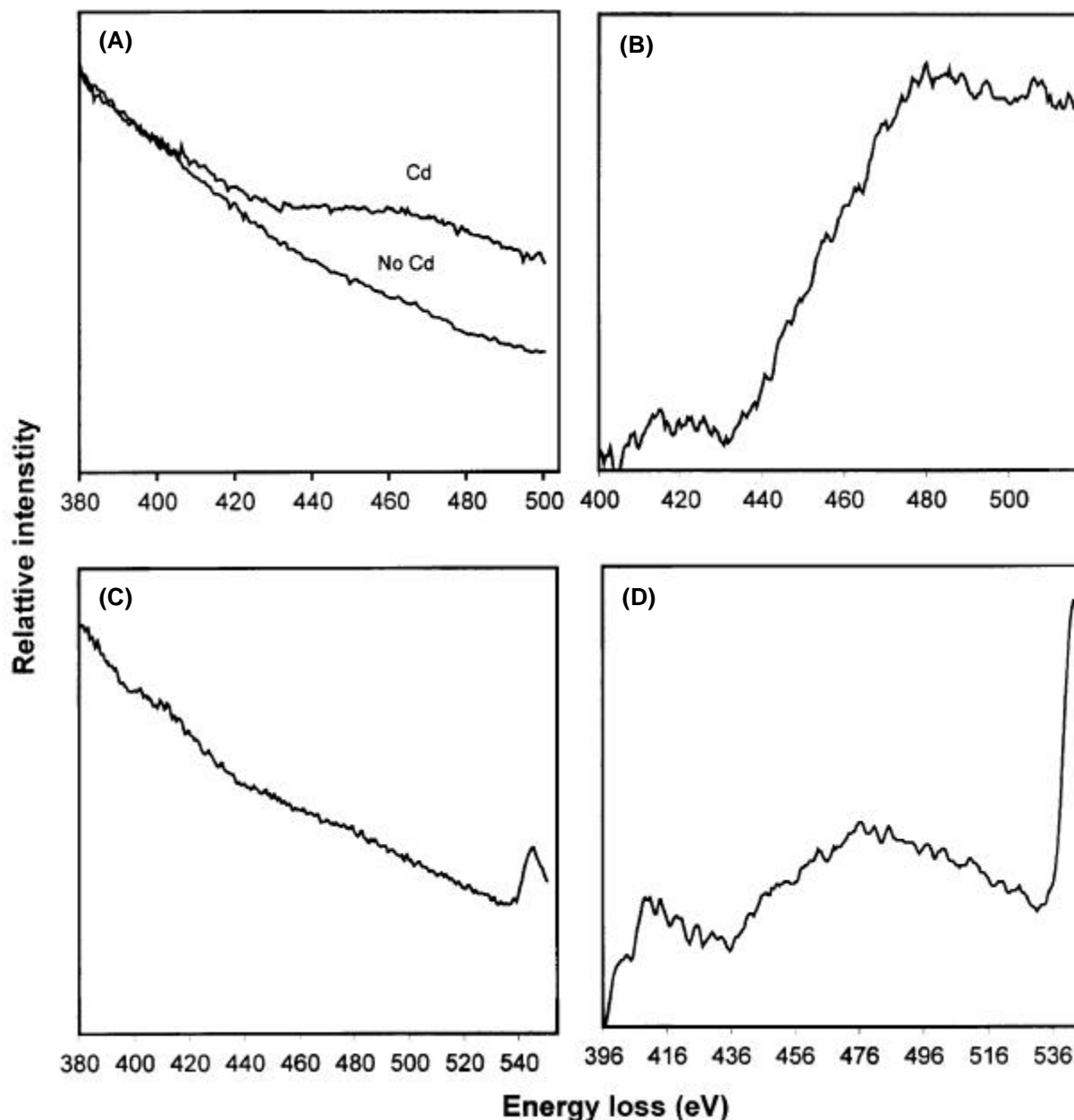
The mechanisms on Cd toxicity in plant cells have been well documented. Generally, plants have a range of potential mechanisms at the cellular level which might be involved in the detoxification of heavy metals and thus tolerance to heavy-metal stress (Hall 2002).

The ultrastructural investigation of root cells of garlic after the treatment with Cd indicated that the structural alternation of root cells and metal accumulation in the cells is dependent on the concentration of the metal. At ultrastructural level, 100  $\mu$ M Cd does not exhibit significant cellular damage to root cells. However, this concen-

tration of Cd causes a greater degree of cell vacuolation, increasing the compartmentation in meristems and cortical parenchyma cells. Sanità and Gabbrielli (1999) indicated that a significant role in Cd detoxification and Cd tolerance is played by vacuolar compartmentalization preventing the free circulation of Cd ions in the cytosol forcing these ions into a limited area. Recent investigations have shown that low Cd concentrations do not however affect plant growth (e.g. Barceló and Poschenrieder 1999). Further, root growth was not significantly affected even by high Cd treatment (Vázquez *et al* 1992a). Our former study showed that cadmium stimulated root length at lower concentrations ( $10^{-5}$ – $10^{-6}$  M Cd) significantly ( $P < 0.005$ ) (Jiang *et al* 2001).

The toxicity symptoms seen in the presence of excessive amounts of Cd may be due to destroying of the defense systems of cells. This paper provides the evidence that at higher concentrations of Cd (1 to 10 mM Cd), the toxic symptoms of root cells are mainly continued to disintegration of cell organelles, disruption of membranes, withdrawal of plasma membrane from cell walls, and formation of multivesiculate bodies in the cytoplasm.

Early researches reported that the occurrence of electron-dense deposits in vacuoles and appearance of small vesicles in cytoplasm seemed to be common features of metal-stressed plants. The presence of metal-bearing granules in the vacuoles and vesicles is related with metal detoxification and tolerance of plants treated with heavy metals (Irmer *et al* 1986; Rauser and Ackerley 1987; Vázquez *et al* 1987; Nassiri *et al* 1997b). Data from EELS in the present investigation confirms that Cd ions are located in electron-dense granules of cells. The level of Cd increases with increase of electron-dense granules. The high content of Cd is detected in vacuoles of cortical cells in differentiating and mature root cells. These results support the findings observed by X-ray microanalysis (Rauser and Ackerley 1987; Vázquez *et al* 1992b), and by analytical EM. These results also support the view that the occurrence of electron-dense deposits in vacuoles and vesicles may play an important role for Cd detoxification by maintaining low levels of Cd (Irmer *et al* 1986; Nassiri *et al* 1997a,b). Storage of Cd as electron-dense granules in nucleoli may be the possible explanation that Cd stimulates the nucleolus to increase production of ribosomes and mRNA. This increase leads to the synthesis of new proteins involved in heavy metal tolerance, and then the formation of sulphhydryl rich phytochelatins may play a role in heavy metal detoxification (Grill *et al* 1987; Tomsett and Thurman 1988; Rauser and Meuwly 1995). These electron dense granules containing Cd are proposed to come from the cell walls where they are enclosed by plasmalemma. Eventually vesicles-like structures are formed. The vesicles, from the cytoplasm to vacuoles, accumulated to be formed into bigger pre-

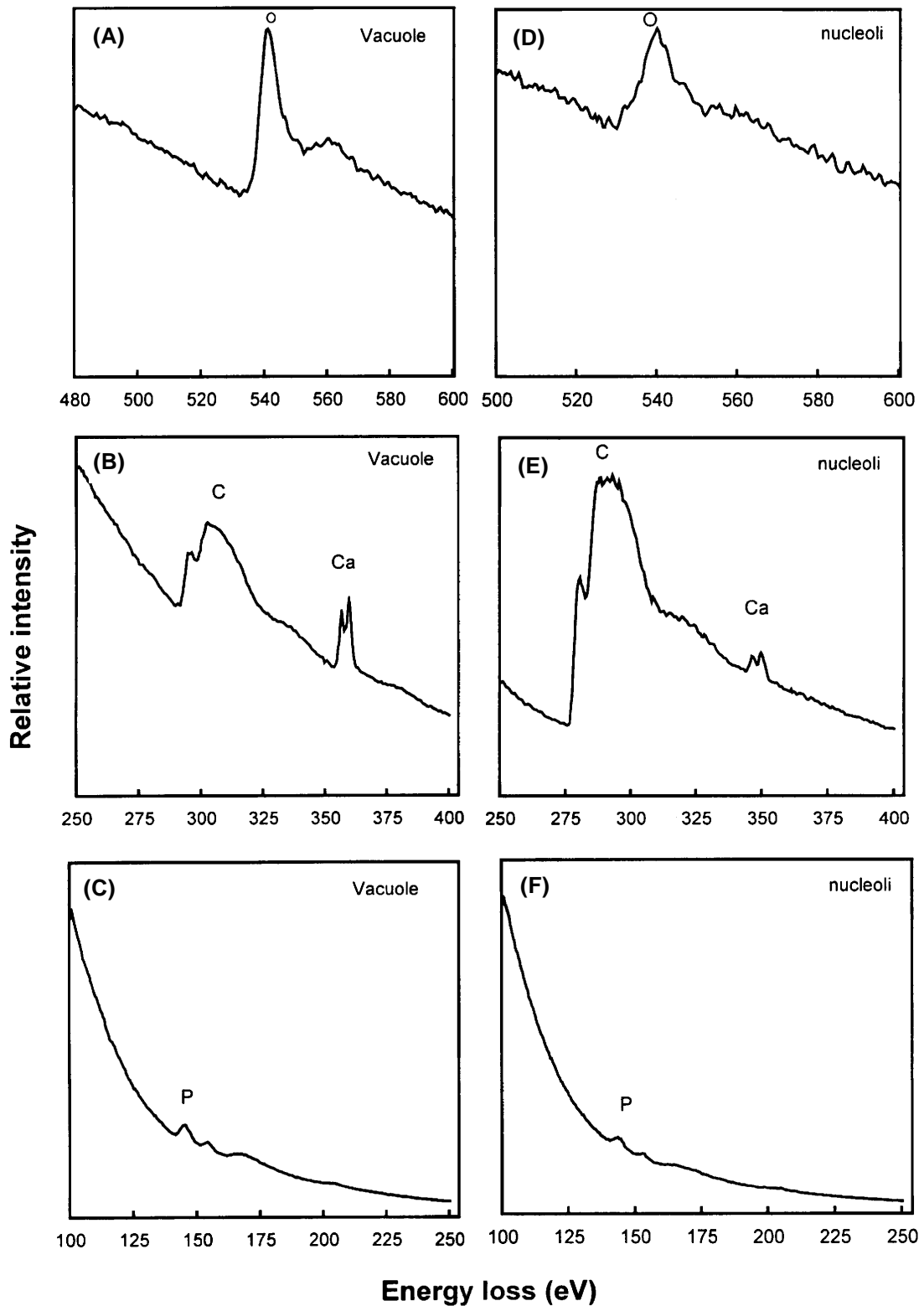


**Figure 2.** Electron energy loss spectra. (A) cadmium spectrum obtained from vacuolar precipitates. (B) 'Stripped' spectrum obtained from vacuolar precipitates containing cadmium. (C) Cadmium spectrum obtained from nucleolar electron-dense granules. (D) 'Stripped' spectrum obtained from nucleolar dense granules containing cadmium after 10 mM treatment for 9 days.

precipitates, with increasing external Cd and prolongation treatment time.

Cell wall was said to play a role in metal tolerance when the cell wall volume is high compared to the cytosol and vacuole (Neumann *et al* 1997). The subcellular localization of Cd in the cell walls has been reported by some authors. However, the results from different authors are quite conflicting. Earlier studies showed that Cd mainly accumulates in cell walls when its content is high

(Lindsey and Lineberger 1981; Khan *et al* 1984; Küpper *et al* 2000), or it mainly accumulates in vacuoles and nuclei (Rauser and Ackerley 1987). Vázquez *et al* (1992a,b), on the basis of their EDXA studies, indicated that while Cd mainly accumulates in cell walls of *Thlaspi caerulescens* roots, Cd was not found in cell wall of *Phaseolus vulgaris* L. roots. In the present work using EELS analytical technology, we clearly showed that no Cd was detected in the cell wall. The conflicting results



**Figure 3.** Electron energy loss spectra. Spectra of O, C, Ca and P obtained from vacuolar precipitates (A–C) and nucleolar electron-dense granules containing Cd (D–F) after 10 mM Cd treatment for 9 days.

may be due to differences between the plant species and between their capacities to accumulate and sequester toxic metals, as well as to differences in the used experimental methods and conditions (Turnau *et al* 1996).

After Cd ions existing in the cytosol, sulphur metabolism is promptly activated, resulting in the production of phytochelatins (Sanità and Gabbrielli 1999). Cd ions bind phytochelatins and form stable PC/Cd complexes and that transported and sequestered in the plant vacuoles (Saxena *et al* 1999). These complexes enter the vacuole and cadmium is released from the phytochelatins, which is then returned to the cytoplasm and the metal may become bound to an organic acid in the vacuole (Greger 1999). Salt *et al* (1996) indicated that the discovery of mechanisms for the transport of both Cd and the Cd-phytochelatin complex across the tonoplast (Salt and Wagner 1993; Salt and Rauser 1995) supports this suggestion that Cd detoxification is achieved by the accumulation Cd, associated with phytochelatins, within the vacuole (Salt *et al* 1995).

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