Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

JZUS

Accumulation and ultrastructural distribution of copper in *Elsholtzia splendens*[*]

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Received Jan. 16, 2005; revision accepted Mar. 16, 2005

Abstract: Copper accumulation and intracellular distribution in *Elsholtzia splendens*, a native Chinese Cu-tolerant and accumulating plant species, was investigated by transmission electron microscope (TEM) and gradient centrifugation techniques. Copper concentrations in roots, stems and leaves of *E. splendens* increased with increasing Cu levels in solution. After exposure to 500 μmol/L Cu for 8 d, about 1000 mg/kg Cu were accumulated in the stem and 250 mg/kg Cu in the leaf of *E. splendens*. At 50 μmol/L Cu, no significant toxicity was observed in the chloroplast and mitochondrion within its leaf cells, but separation appeared at the cytoplasm and the cell wall within the root cells. At >250 μmol/L Cu, both root and leaf organelles in *E. splendens* were damaged heavily by excessive Cu *in vivo*. Copper subcellular localization in the plant leaf after 8 days' exposure to 500 μmol/L Cu using gradient centrifugation techniques was found to be decreased in the order: chloroplast>cell wall>soluble fraction>other organelles. The plant root cell wall was found to be the site of highest Cu localization. Increase of Cu exposure time from 8 d to 16 d, increased slightly Cu concentration in cell wall fraction in roots and leaves, while that in the chloroplast fraction decreased in leaves of the plants grown in both 0.25 μmol/L and 500 μmol/L Cu. TEM confirmed that much more Cu localized in cell walls of *E. splendens* roots and leaves, but also more Cu localized in *E. splendens*' chloroplast when the plant is exposed to Cu levels>250 μmol/L, as compared to those in the plant grown in 0.25 μmol/L Cu. Copper treatment at levels>250 μmol/L caused pronounced damage in the leaf chloroplast and root organelles. Copper localization in cell walls and chloroplasts could mainly account for the high detoxification of Cu in *E. splendens*.

Key words: Cell wall, Chloroplast, Cu detoxification, *Elsholtzia splendens*, Ultrastructural distribution
doi:10.1631/jzus.2005.B0311 **Document code:** A **CLC number:** X53

INTRODUCTION

Copper is an essential nutrient for plant growth and development, although it is highly phytotoxic at excessive levels. Mining, smelting and land applications of sewage sludge, together use of fungicides containing Cu, and other human activities, lead to widespread soil contamination with copper. Plants growing on Cu contaminated soils develop resistance to Cu in the soil and detoxify Cu inside the plant cell (Ernst *et al*., 1992; Macnair, 1993). *E. splendens* is reportedly a native Chinese Cu-tolerant and accumulating plant species based on mined areas investigation and greenhouse hydroponics and pot experiments (Yang *et al*., 1998; Song *et al*., 2004). It can survive normally in soil contaminated with more than 3000 mg/kg Cu (Tang *et al*., 1999), suggesting the existence of defense mechanisms in *E. splendens* against the harmful effects induced by copper toxicity.

Many studies had been carried out on the effect of copper on the growth, mineral nutrition and metabolism of plants. Copper excess reduces plant growth (Maksymiec *et al*., 1995), phytosynthetic activity (Lidon *et al*., 1993) and the quantum yield of

[*] Project supported by the National Natural Science Foundation of China (No. 20307008) and the National Basic Research Program (973) (No. 2002CB410804) of China

PSII phytochemistry which was detected by chlorophyll fluorescence (Maksymiec and Baszynski, 1999). Copper excess may also result in membrane damage by Cu binding to the sulfhydryl groups of membrane proteins (Kennedy and Gonsalves, 1987). For most crop species, the critical level for copper toxicity in leaves is above 20−30 mg/kg dry weight (Robson and Reuter, 1981). In plant cells under normal conditions, free copper is virtually nonexistent as the cell has an overcapacity for copper sequestration (Rae *et al*., 1999). However, under conditions of copper overload, free copper ions can accumulate and react to generate hydroxyl radicals participating in reactions that can adversely modify proteins, lipids, and nucleic acids (Halliwell and Gutteridge, 1984). Many metal tolerant organisms are also accumulators of metals. Therefore, other mechanisms of avoiding the toxic effects of accumulated metal may induce metal sequestration in organelles or metal complexation by metal-binding molecules or metal precipitation.

In this study, the patterns of Cu localization and subcellular distribution within the root and leaf cells of *E. splendens* at different Cu levels were studied by TEM and different speed centriguation techniques for further elucidating Cu intracellular detoxification in *E. splendens*.

MATERIALS AND METHODS

E. splendens seeds, collected from adult plants growing on copper mining deposit in Zhuji County of Zhejiang Province of China, were germinated as described by Yang *et al*.(2002) until 3-week seedlings were observed. The uniform 3-week seedlings were transferred to a nutrient solution for 14 d pre-culture (6-leaf seedlings). High Cu (500 μmol/L) and low Cu (0.25 μmol/L) treatments were applied, added as $CuSO_4·5H_2O$, the plants were grown for 16 d in the greenhouse. Plants were harvested at day 8 and 16 of treatment for assay of Cu subcellular distribution.

For TEM assay, younger seedlings (4-leaf seedlings) were selected for Cu treatments: with 0.25, 50, 100, 250 and 500 μmol/L, added as $CuSO_4·5H_2O$, and the plants were grown for 8 d in the greenhouse.

The composition of the full strength nutrient solution was (in μmol/L): 700 K_2SO_4, 100 KCl, 2000 $Ca(NO_3)_2·4H_2O$, 500 $MgSO_4·7H_2O$, 100 KH_2PO_4, 10 H_3BO_3, 0.5 $MnSO_4·H_2O$, 0.5 $ZnSO_4·7H_2O$, 0.2 $CuSO_4·5H_2O$, 0.01 $(NH_4)_6Mo_7O_{24}$, 100 Fe-EDTA. The experiment was randomly arranged with three replicates for each treatment. The solution was aerated and maintained at pH 5.8±0.3 adjusted daily with 0.1 mol/L NaOH or 0.1 mol/L HCl, and was renewed every 4 d during the experiment. All experiments were conducted in a greenhouse with the temperature ranging from (28±2) °C (day) to (15±2) °C (night), without supplementary artificial light.

At harvest, roots of intact plants were rinsed with distilled water, and then immersed in 5 mmol/L $Pb(NO_3)_2$ for 20 min to remove the putative adsorbed Cu^{2+} (Harrison *et al*., 1984), then for TEM assays, subcellular distribution of Cu, and metal analysis.

1. TEM assay: small sections of leaves and roots, 1−3 mm in length, were fixed in 4% glutaraldehyde (v/v) in 0.2 mol/L PBS (sodium phosphate buffer, pH 7.2) for 6−8 h and post-fixed in 1% OsO_4 [Osmium (VIII) oxide] for 1 h, then in 0.2 mol/L PBS (pH 7.2) for 1−2 h. Dehydration was performed in a graded ethanol series (50%, 60%, 70%, 80%, 90%, 95% and 100%) followed by acetone, then samples were filtrated and embedded in Spurr's resin. Ultra-thin sections (80 nm) were prepared and mounted on copper grids for viewing under transmission electron microscope (JEOL TEM-1200EX) at an accelerating voltage of 60.0 kV or 80.0 kV.

2. Subcellular distribution of Cu: fresh samples of plant roots and leaves of high Cu (500 μmol/L) and low Cu (0.25 μmol/L) were selected after 8 d and 16 d, weighed and ground in a quartz mortar with 10 mmol/L Tris-HCl buffer solution (pH 7.4, containing 2.5% ascorbic acid) added at the ratio of 1:5 according to the method of Brooks *et al*.(1981). Cells were separated into different fractions: cell wall, chloroplast, nucleus, mitochondrion, ribosome and soluble fraction by gradient centrifugation technique at 4 °C (J2-HS, BECKMAN), as shown in Fig.1. The different cell fractions obtained were oven-dried at 65 °C, ashed at 550 °C for 6 h, and dissolved in 1:1 (v:v) HNO_3, then the subcellular fractions of Cu in root and leaf of *E. splendens* were estimated by ICP-OES (Model IRAS-AP, TJA).

3. Metal analysis: roots, stems and leaves of *E. splendens* were separated, washed with distilled water, and oven-dried at 65 °C. The dried plant materials

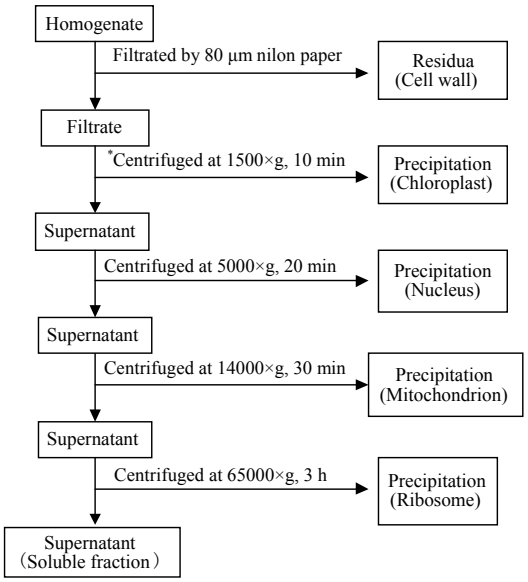

Fig.1 Schematic of the separation of the subcellular fractions by differential speed centrifugation (*for root: centrifuged at 2500×g for 20 min)

were ground in a stainless steel mill and passed through a 0.25-mm sieve, then ashed at 550 °C for 6 h, and dissolved in 1:1 (v:v) HNO_3. Copper concentrations in the plant digests were analyzed by ICP-OES (Model IRAS-AP, TJA).

All the data were analyzed by SPSS (Version 11.0) with three replicates. One-way ANOVA was employed to evaluate whether the means were significantly different at $P<0.01$.

RESULTS

Copper accumulation in *E. splendens*

Copper levels in the roots, stems and leaves of the plants increased at increased Cu levels in the nutrient solution, and Cu distribution in the plant organs were: root>>stem>leaf (Fig.2). After Cu exposure for 8 d, slightly increased Cu in the stems and leaves of *E. splendens* were noted at 50 μmol/L Cu, and it significantly increased at >100 μmol/L Cu as compared to that at 0.25 μmol/L Cu. At 500 μmol/L Cu, stem Cu was about 1000 mg/kg, and leaf Cu was about 250 mg/kg.

Transmission electron microscopy

In a section of the *E. splendens* leaf (3rd leaf from the top) of the control, multiple chloroplasts were

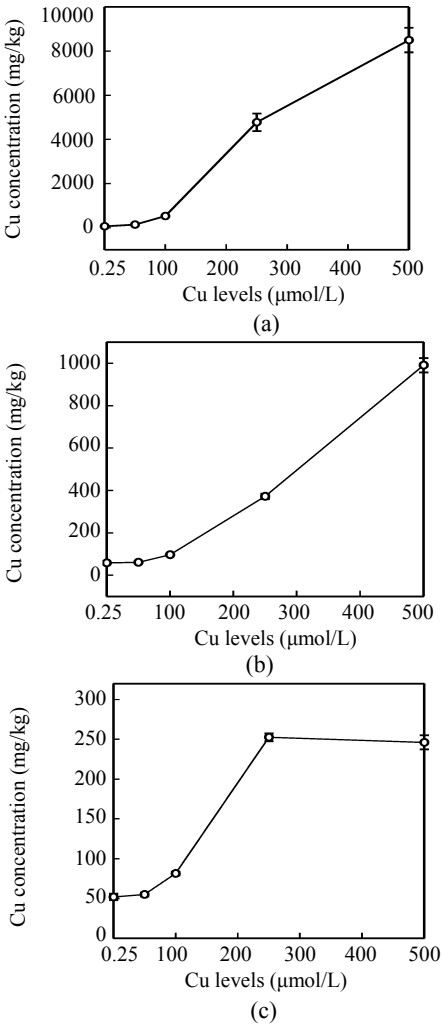

Fig.2 Cu concentration in (a) roots, (b) stems and (c) leaves of *E. splendens* at different Cu levels for 8 d. All the data are the means of 3 replications

within the leaf cells, and the thylakoid array in chloroplast paralleled the long axes. Numerous plastoglobuli and starch grain were evident within the chloroplasts. Small cristates in the mitochondria arrayed closely (Fig.3a). At 50 μmol/L Cu, some thylakoid arrays in the chloroplast were tortuous. The morphology of chloroplast and mitochondrion were intact. The cytoplasm was not separated from the cell wall, the nucleus structure was intact (Fig.3b). At 250 μmol/L Cu, the chloroplasts were not spherical, and the thylakoid array structure became smaller and was damaged heavily. The mitochondrion was damaged heavily, and zigzag cytoplasms were noted (Fig.3c). At 500 μmol/L Cu, the membranes in the chloroplast, mitochondrion and the cytoplasm were damaged heavily (Fig.3d), the thylakoid arrays disassembled

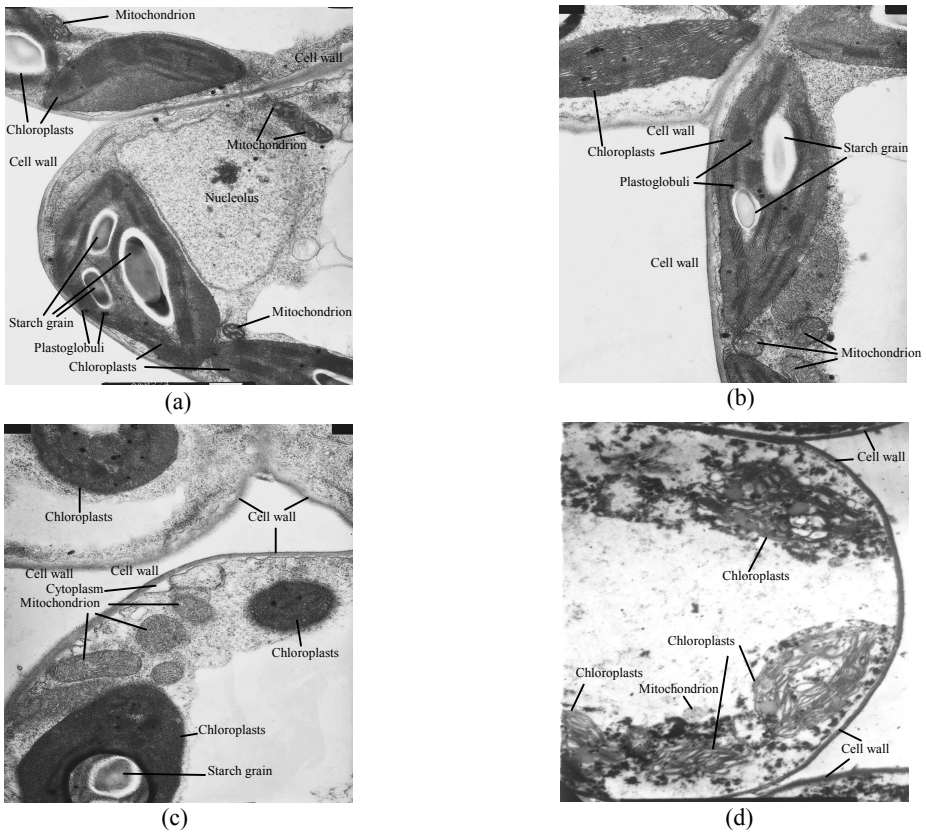

Fig.3 TEM photos of *E. splendens* leaf cell (a) Control (×10000); (b) 50 μmol/L (×15000); (c) 250 μmol/L (×12000); (d) 500 μmol/L (×8000)

and chloroplasts were damaged heavily. Large amounts of copper were intensively deposited in the chloroplast membrane and the cell wall. These observations indicated that, as the Cu increased to 500 μmol/L, the chloroplast was adversely affected by excessive Cu. At 50 μmol/L Cu, no significant toxicity was observed in the chloroplast and the mitochondrion within the leaf cells of *E. splendens*, as compared to the control. While at 500 μmol/L Cu, the leaf organelles of *E. splendens* were damaged heavily by excessive Cu *in vivo*.

In the control, the multiple mitochondrions within the root cells were intact, most cristates in the mitochondrion double-deck membranes were evenly distributed except for small portions that were distributed unevenly (Fig.4a). At 50 μmol/L Cu, some damage was noted in the mitochondrion membranes (Fig.4b). Separation appeared between the cytoplasm and the cell wall, most of the containers were distributed unevenly, with cristates disappearing from them except for the small portions in containers that were kept intact (Fig.4c). The structure of nucleus and of nucleolus was kept intact as compared to those of the control, and many more vacuoles were within the root cell (Fig.4d). At 100−250 μmol/L Cu, the root organelles were heavily damaged (Figs.4e and 4f). Hollowed root cells appeared, big dark copper particles precipitated within the root cells and saturated at the root cell walls (Fig.4e) and at the root cell membrane (Fig.4f). Compared to the nucleus in the root cells of *E. splendens*, the mitochondrions are more easily destroyed by excessive Cu *in vivo*.

Copper subcellular localization in the plant cell

Table 1 of Cu subcellular localization in leaf cells of *E. splendens* shows that Cu contents in nucleus, mitochondrion and ribosome of plants changed slightly, while that in the cell wall, chloroplast and soluble fraction increased, when the plant was exposed to 0.25 μmol/L Cu from 8 d to 16 d. At 500 μmol/L Cu, the Cu content in all organelles and the soluble fraction in the plant leaf cells was elevated as the Cu exposure time was increased from 8 d to 16 d. Furthermore, the Cu content in all organelles and the

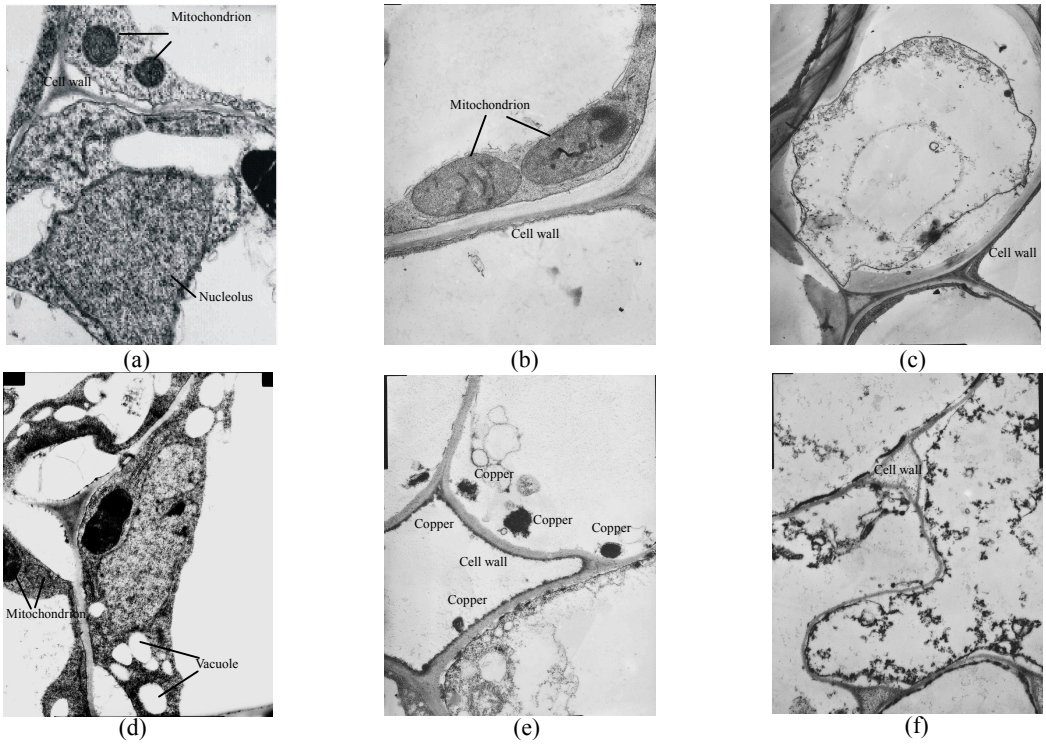

Fig.4 TEM photos of *E. splendens* root cell (a) Control (×12000); (b) 50 μmol/L (×15000); (c) 50 μmol/L (×6000); (d) 50 μmol/L (×10000); (e) 250 μmol/L (×10000); (f) 100 μmol/L (×10000)

soluble fraction in the leaf cells of plants increased with the increase in Cu levels. When the Cu exposure time was increased from 8 d to 16 d, the distribution percent of Cu in the chloroplast, mitochondrion, nucleus and ribosome at 0.25 μmol/L Cu decreased, and was slightly changed in the nucleus, mitochondrion and ribosome. A decreased percent in chloroplast was noted at 500 μmol/L Cu. The Cu distribution percent in chloroplast, nucleus, mitochondrion and ribosome decreased but in the soluble fraction and cell wall in the plant leaf cells increased as increasing Cu levels. In short, the Cu subcellular localization in the leaf cells of *E. splendens* is uneven, and in the order of chloroplast>cell wall>soluble fraction>ribosome> mitochondrion>nucleus at 0.25 μmol/L Cu, but at 500 μmol/L Cu, the order was: cell wall>chloroplast> soluble fraction>ribosome>mitochondrion>nucleus, which may be due to the increased Cu bonded to the cell wall in the plant leaf cells under high Cu toxic condition.

The subcellular localization of Cu in the root cells of *E. splendens* is shown in Table 2. There was no notable change in Cu absolute contents in nucleus, mitochondrion, ribosome and soluble fractions in plant root cells, when Cu contents in the cell wall fractions increased after the plant was exposed to 0.25 μmol/L Cu for 8 d to 16 d. While at 500 μmol/L Cu, the Cu contents in all organelles rose considerably as the Cu exposure time increased from 8 d to 16 d. Elevated Cu levels increased Cu contents in all organelles in the root cells of the plants. When Cu exposure time increased from 8 d to 16 d, the Cu distribution percent in nucleus, mitochondrion, ribosome and soluble fraction decreased at both 0.25 μmol/L and 500 μmol/L Cu, that in cell wall fraction increased and in plastid fraction changed slightly at 500 μmol/L Cu. In short, the order of Cu subcellular localization in root cells of *E. splendens* was: cell wall>plastid>soluble fraction>ribosome>mitochondrion=nucleus at both 0.25 μmol/L and 500 μmol/L Cu.

DISCUSSION AND CONCLUSION

Much more Cu was located at the roots than shoots of *E. splendens* (Fig.2) after Cu exposure for 8 d. Fifty μmol/L Cu slightly increased Cu in the stems

Table 1 Copper subcellular distribution in the leaf cell of plants exposued to 0.25 and 500 μmol/L Cu (mg/kg FW)

Fractions	Cu subcellular localization			
	0.25 μmol/L Cu (control)		500 μmol/L Cu	
	8 d	16 d	8 d	16 d
Cell wall	8.6 (29)*	11.9 (31)	45.6 (37)	58.4 (38)
Chloroplast	13.9 (46)	16.6 (43)	47.5 (38)	51.4 (34)
Nucleus	1.2 (4.0)	1.3 (3.4)	2.8 (2.3)	3.7 (2.4)
Mitochondrion	1.5 (5.0)	1.6 (4.2)	4.6 (3.7)	5.15 (3.4)
Ribosome	2.3 (7.7)	2.7 (7.0)	5.23 (4.2)	6.13 (4.0)
Soluble fraction	2.5 (8.3)	4.2 (11)	18.2 (15)	27.3 (18)
Total Cu	35.5	43.8	137	163
Percentage recovery (%)#	85	87	90	93

*Values in the bracket represent distribution percent (%); #Percentage recovery (%)=(Cell wall+Chloroplast+Nucleus+Mitochondrion+Ribosome+Soluble fraction)/Total

Table 2 Copper subcellular distribution in the root cell of plants exposued to 0.25 and 500 μmol/L Cu (mg/kg FW)

Fractions	Cu subcellular localization			
	0.25 μmol/L Cu (control)		500 μmol/L Cu	
	8 d	16 d	8 d	16 d
Cell wall	7.82 (47)*	8.75 (45)	1038 (56)	2380 (61)
Plastid	4.17 (25)	5.69 (29)	265 (14)	558 (14)
Nucleus	0.91 (5.6)	1.05 (5.3)	90.3 (4.9)	162 (4.2)
Mitochondrion	0.96 (5.7)	1.02 (5.2)	84.7 (4.6)	126 (3.2)
Ribosome	1.12 (6.7)	1.27 (6.5)	83.2 (4.5)	103 (2.6)
Soluble fraction	1.85 (11)	1.91 (9.7)	295 (16)	573 (15)
Total Cu	19.6	23.5	2024	3804
Percentage recovery (%)#	86%	84%	92%	103%

*Values in the bracket represent distribution percent (%); #Percentage recovery (%)=(Cell wall+Plastid+Nucleus+Mitochondrion+Ribosome+Soluble fraction)/Total

and leaves of *E. splendens*, while it was significantly increased at Cu levels>100 μmol/L. At 500 μmol/L Cu, stem Cu was around 1000 mg/kg, and leaf Cu was approximately 250 mg/kg. TEM observations confirmed that chloroplasts was damaged more heavily than other organelles in leaf cell of *E. splendens* when Cu levels increased up to 500 μmol/L in nutrient solution. At 50 μmol/L Cu, no significant toxicity was observed in the chloroplast and the mitochondrion within the leaf cells of *E. splendens*, as compared to the control. Whereas at 250 μmol/L Cu, chloroplasts deviated considerably from spherical shape and thylakoid arrays decreased significantly, plasmalemma exhibited zigzag pattern. At Cu levels up to 500 μmol/L, the organelles in leaf cells of *E. splendens* were damaged heavily by excessive Cu *in vivo*. Large amounts of electron dense bodies (copper particles) were deposited near the inner side of cell wall, at the outer side of the chloroplast membrane and within the chloroplast (Fig.3). TEM photos of *E. splendens* root cells showed that some damage occurred in the mitochondrion membranes, separation of the cytoplasm from the cell wall was noted at 50 μmol/L Cu (Fig.4b). At 100−250 μmol/L Cu, the root organelles were heavily damaged, and big dark copper particles precipitated within the root cell and saturated at the outer side of the root cell walls (Fig.4e) and at the root cell membrane (Fig.4f). But the structure of the nucleus and of the nucleolus was intact. Compared to the nucleus in the root cells of *E. splendens*, the mitochondrions can be more easily destroyed by excessive Cu *in vivo*.

Copper subcellular localization in the plant's leaf cell after 8 days' exposure of the plant to 500 μmol/L Cu decreased in the order: chloroplast>cell wall>soluble fraction>other organelles. Whereas for the plant root cell, cell wall was the highest Cu localization site, followed by the plastid and the soluble

fraction, and the lowest in the other organelles. The chloroplasts, nucleis, mitochondrions and ribosomes are the key cell organs in the plant cell, for the major cell life activities (Carroll, 1989; Westerhoff, 1985). Copper has strong affinity to the functional groups like sulfhydryl in the organelle membrane. At proper levels, Cu can keep the structure steady in the organelle membrane (Henriques, 1989), while at excessive levels, it can damage the integrity of the membrane structure within the plant cell (De Vos *et al*., 1989; 1991). At 500 μmol/L Cu, the Cu distribution percent in organelles in the root and leaf cells decreased, but that in the cell wall and soluble fraction increased, as compared to that at 0.25 μmol/L Cu. The decrease in Cu distribution percent observed at exposure concentrations that affect the structure of many organelles may just be due to effects on transport systems. For the same Cu exposure level and exposure time, Cu is mainly deposited in the cell wall, then chloroplast and the soluble fraction in the plant leaf cells. At 0.25 μmol/L Cu, chloroplast is the main Cu localization site in plant leaf cells for the normal requirements for plant growth (Lastra *et al*., 1987). After increasing Cu to 500 μmol/L, the distribution percent of Cu in the cell wall and chloroplast was even at 8 d, but after the plant's exposure to Cu for 16 d, increased distribution percentage in the cell wall and decreased distribution percentage in the chloroplast were noted in the plant leaf cells (Table 1).

The uneven increase of distribution percent and Cu absolute content in cell wall of plant root cells were observed as the Cu in solution increased (Table 2). Root cell wall is the important localization site of heavy metals in plants due to its quantities of cation ligand (Hayens, 1980; Leita *et al*., 1996). In this study, 60%–70% root Cu localized at the root cell wall, which accorded with reports that over 50% root Cu is bonded to the root cell wall of plants (Cathala and Salsac, 1975; Iwasaki *et al*., 1990). Nishizono *et al*.(1989) reported that about 70%–90% Cd, Cu and Zn in the root cell of *Athyrium yokosense* were bonded to the root cell wall. The plastid can be the main storage organelle in the plant root because of its role as the precursor of chloroplast, but its function cannot be equivalent to that of the chloroplast (Carroll, 1989). The Cu distribution percent in the plastid significantly decreased at 500 μmol/L Cu when Cu exposure time was extended from 8 d to 16 d. Moreover, at the high Cu level, the soluble fraction that increased markedly, resulted mainly from the enhanced syntheses of Cu-complex of low molecular weight in the protoplast of plant (Krotz *et al*., 1989; Wagner and Krotz, 1989).

Plant cell wall is the main composition of apoplasts, which are the "dead" tissues in the plants with lower physiological metabolism activity. The plant cell wall contains protein and polyoses such as cellulose, hemicellulose, and lignin, mucilage glue, and so on, which have a number of potential ligands such as hydroxyl, carboxyl, amino group, aldehyde group, phosphate, thiol, etc. (Hayens, 1980) that can participate in a variety of reactions including ion exchange, adsorption, complexation, precipitation and crystallization, leading to metal sequestration under metal toxicity (Mullen *et al*., 1992). When exposed to higher levels of metals, the plant cell can actively secrete calluses which have the ability of chelation to the apoplast parts (Wissenmeier *et al*., 1987). So, the plant cell wall is the chief site for detoxification of heavy metals in plant (Hayens, 1980; Allan and Jarrell, 1989). When plants were exposed to nonlethal levels of Ni, 70% Ni in the Ni-hyperaccumulator *Thlaspi goesingense* was combined with cell wall substances (Krämer *et al*., 2000). In this study, at 500 μmol/L Cu, the plant cell wall is the main Cu location site both in the leaf and root cell of *E. splendens*. While in the leaf cell, chloroplast was the other important Cu location site. At exposure to 500 μmol/L Cu or with the longer Cu exposure time, Cu location in the cell wall increased considerably, and that in the chloroplast decreased markedly. The Cu localization in the cell walls and chloroplasts could mainly account for the high detoxification of Cu in *E. splendens*.

ACKNOWLEDGEMENTS

The authors thank Dr. J.Y. Li from the Center of Analysis & Measurement of Zhejiang University for her kind help in the transmission electronic microscope analyses of the plant samples.

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