

Copper Uptake and Its Effect on Metal Distribution in Root Growth Zones of *Commelina communis* Revealed by SRXRF

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Abstract To explore the copper uptake mechanisms by the Cu-tolerant plant *Commelina communis*, the contents of Cu and other metals (including Fe, Zn, and Mn) in roots were detected using atomic absorption spectrometer under transporter inhibitors, partial element deficiency, or Cu excess treatments, while distribution characters of Cu and other metals in root growth zones were investigated by synchrotron radiation X-ray fluorescence spectroscopy (SRXRF). Cu uptake was inhibited by the uncoupler DNP and P-type ATPase inhibitor Na_3VO_4 , not by the Ca^{2+} ion channel inhibitor LaCl_3 , suggesting that Cu could probably be assimilated actively by root and be related with P-type ATPase, but not through Ca^{2+} ion channel. Fe or Zn deficiency could enhance Cu uptake, while 100 μM Cu inhibited Fe, Zn, and Mn accumulation in roots significantly. Metal distribution under 100 μM Cu treatment was investigated by SRXRF. High level of Cu was found in the root meristem, and higher Cu concentrations were observed in the vascular cylinder than those in the endodermis, further demonstrating the initiative Cu transport in the root of *C. communis*. Under excess Cu stress, most Fe was located in the epidermis, and Fe concentrations in the endodermis were higher than those in the vascular cylinder, suggesting Cu and Fe competition not only in the epidermal cells but also for the intercellular and intracellular transport in roots. Zn was present in the meristem and the vascular cylinder similar to Cu. Cu and Zn showed a similar pattern. Mn behaves as Zn does, but not like Fe.

Keywords *Commelina communis* · Copper uptake · Distribution · Root growth zones · SRXRF

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Introduction

Copper is an essential element for normal plant metabolism and has also been reported to be among the most toxic metals [1]. Accumulation of Cu in soils results from various sources including the following: the use of fertilizers, the application of pig and poultry slurries rich in Cu, fungicides, atmospheric deposition from industrial and urban activities, metaliferous mining, or metal processing, which inhibits plant growth and development [2, 3].

Plants can accumulate heavy metals in different plant tissue or cell organelles [4–6] and also can detoxify heavy metals by chelating them to organic acids or proteins [7, 8]. The roots are the sole organs penetrating the soil and thus the ones to be in direct contact with the toxic Cu. It is, therefore, expected that roots are the first to develop the responding symptoms. Studies suggested that much of the Cu accumulated in root tissue is cell wall associated [9]. The casparian strip and endodermis seems to provide selectivity against Cu, with reduced concentrations entering the stele of root [10]. Ni et al. [11] found that most Cu in protoplasts of *Elsholtzia splendens* was compartmented in the vacuoles. In some plants, Cu tolerance was related to metallothioneins (MTs) and phytochelatins (PCs) [8, 12, 13].

Metal interactions affecting distribution have been noted in various species of plants with additional metals having both positive and negative effects on uptake and deposition of a given metal [14]. The increasing soil Cu concentration was found in oregano to relate to increasing root bio-accumulation of Cu, Fe, and Mn and decreasing of Ca, K, and Zn, respectively [15]. Fe deficiency in the growth medium increased Cu concentration in maize [16] and pea [17]. Fe increase in the nutrient medium suppressed Cu concentration in apple plants [18]. Lin and Wu [19] found that in *Lotus purshianus*, elevated Cu in soil reduced Zn uptake. However, Luo and Rimmer [20] found that copper increased the uptake of Zn by barley. Bernal et al. [3] reported foliar and root Cu supply affecting differently Fe and Zn uptake in soybean plants. All these studies demonstrated that cation homeostasis in the root will be controlled by metal interactions.

Commelina communis is a Cu-tolerant plant that grows in copper mine areas along the middle and lower streams of the Yangtze River [21, 22]. However, its mechanisms of Cu uptake and tolerance need further elucidation. Many techniques have been studied for the element localization and heavy metal tolerance mechanisms in plants such as electron dispersive X-ray spectroscopy, electron energy loss spectroscopy, and proton-induced X-ray analysis. Compared with these techniques, synchrotron radiation X-ray fluorescence spectroscopy (SRXRF) microprobe is more sensitive and less injurious to cells and also has excellent characteristics such as very high brightness, collimation, polarization, and lower bremsstrahlung, which is the electromagnetic radiation produced by an accelerated electrically charged subatomic particle. Up to now, SRXRF has been extensively applied in many fields such as materials science, life science, medical science, and environmental science [23, 24]. In this study, we investigated the copper uptake mechanisms in root of *C. communis* under transporter inhibitors and partial Fe or Zn deficiency treatments and revealed Cu and other metal distribution characters in root growth zones by SRXRF.

Materials and Methods

Plant Material and Culture

The seeds of *C. communis* were collected from the Huajiachi campus of Zhejiang University, China. They were soaked in distilled water overnight and then germinated on

moistened cellulose tissue in a porcelain dish at 25°C. After germination, the solution was changed to one-fourth strength of complete nutrient solution. After 10 days, the uniform seedlings were transferred to vessels filled with full-strength nutrient solution containing macronutrients [1.0 mM $\text{Ca}(\text{NO}_3)_2$, 0.5 mM MgSO_4 , and 0.5 mM K_2HPO_4] and micronutrients (14.0 μM H_3BO_3 , 3.0 μM MnSO_4 , 0.3 μM Na_2MoO_4 , 0.5 μM CuSO_4 , 16.0 μM Fe-EDTA, and 0.3 μM ZnSO_4). The pH of solution was adjusted to 6.0. Nutrient solutions were renewed every 3 days. Plants were grown in a controlled environment growth chamber with a 16-h, 25°C day and an 8-h, 20°C night regime, approximately 10 klx light intensity, and 60–70% relative humidity. The 40-day-old plants were subjected to transporter inhibitors and partial Cu, Fe, and Zn deficiency and Cu excess treatments. Each treatment was maintained with three replicates.

Metal Uptake Experiments and Content Analyses

The seedlings were treated with 50 μM 2,4-dinitrophenol (DNP) (the uncoupler of oxidative phosphorylation) or 50 μM Na_3VO_4 (the P-type ATPase inhibitor) for 5 h in nutrient solutions containing 5 or 20 μM CuSO_4 . In addition, the seedlings were treated with 0.1 mM LaCl_3 (the Ca^{2+} ion channel inhibitor) for 48 h in the nutrient solutions containing 5 or 20 μM CuSO_4 .

When plants were 40 days old, Fe or Zn deficiency was induced, replacing the full nutrient solution with a nutrient solution without Fe or Zn for 6 days and then seedlings were treated by 5 or 20 μM CuSO_4 under Fe or Zn deficiency and were subsequently harvested after 48 h uptake.

Cu deficiency was induced, replacing the full nutrient solution with a nutrient solution without Cu for 6 days, and then the seedlings were harvested for metal determination. In addition, seedlings were treated by 0.5 (control), 5, 20, 50, 100, and 200 μM CuSO_4 for 15 days, and then samples of roots were taken for metal content analyses. Subsamples of 100 μM CuSO_4 -treated roots were used for SRXRF measurements.

The roots of harvested seedlings were desorbed with 5 mM $\text{Pb}(\text{NO}_3)_2$ for 15 min and then rinsed thoroughly with deionized water and dried at 80°C. Subsamples of ground tissue were weighed (0.5 g) and digested with HNO_3 and HClO_4 ($\text{HNO}_3:\text{HClO}_4=4:1$). The clear digest samples were then transferred quantitatively to 25 mL calibrated flasks with deionized water, and the metal concentrations were determined using atomic absorption spectrometer. Blanks and a standard reference material were analyzed concurrently for accuracy assurance. Each sample was in triplicate.

Metal Distribution Detected by SRXRF

The 100- μM CuSO_4 -treated roots were cut off from plant and then frozen by liquid nitrogen. A longitudinal section (50 μm thick) around meristem and cross sections (50 μm thick) in elongation tissue (around 0.5 cm above the root apex) and in non-growing tissue (around 5 cm above the root apex) were cut with a cryotome (HM505E, MICROM CO.) at a temperature of -20°C and subsequently freeze-dried.

The SRXRF experiments were performed at the XRF microprobe station in Beijing Synchrotron Radiation Facility (BSRF). The X-ray light source comes from the 4W1B beam line at BSRF. This beam line can provide multi-chromatic X-rays (white light), whose energy range from 3.5 to 35 keV. The electron energy in the storage ring is 2.2 GeV, with a current range from 70 to 140 mA. The XRF microprobe experimental station lies 25 m away from the point of the light source. Because the emission angles of the light in

horizontal and vertical directions are only 1.0 and 0.1 mrad, respectively, this high collimation confers superior performance on the synchrotron radiation XRF microprobe. XRF spectra were collected using a PGT Si (Li) solid detector, positioned at 90° to the beam line, 7 mm from the target. The apparatus of SRXRF in BSRF was reported in detail by Huang et al. [25]. During the SRXRF scanning experiments, the spot size of the X-ray beam was adjusted to 30×30 μm². The scanning dot moved according to the displacement ΔX=50 μm, ΔY=50 μm, and each dot had been scanned for 60 s. The scanning areas of sections from different root growth tissue are shown in Fig. 6. All the SRXRF spectra were analyzed by AXIL.

Statistical Analysis

The data were statistically analyzed with one-way analysis of variance (ANOVA) using the SPSS 16.0 for Windows. The results were expressed as means of three replicates. Bars in the figures indicated standard deviation (SD) of the data. A probability level of 0.05 was considered to be statistically significant.

Results

Cu Uptake by Root under Inhibitor or Fe and Zn Deficiency

DNP is known as an uncoupler of oxidative phosphorylation, while vanadate is thought to inhibit P-type ATPases by blocking the shift in conformation states during the reaction cycle of these enzymes [26]. The influence of DNP and Na₃VO₄ on Cu uptake was shown in Fig. 1. Cu concentrations in roots decreased when adding DNP and Na₃VO₄ into solution containing 5 or 20 μM Cu ($P<0.05$), which showed proper initiative Cu uptake in *C. communis*. The Ca²⁺-ATPases inhibitor LaCl₃ did not inhibit Cu uptake in solution containing 5 or 20 μM Cu (Fig. 2), suggesting that Cu uptake by *C. communis* was not through Ca²⁺ ion channel.

Under micronutrient deficiency conditions, induction of high-affinity transporters occurs, whereas under toxic conditions, efflux pumps or sequestration into intracellular compartments serves to restrict the concentration in the cytoplasm [27]. Cu concentrations in Fe deficiency root were 2.9-fold or 3.7-fold higher than that in the control root, when the seedlings were treated with 5 or and 20 μM CuSO₄, respectively (Fig. 3). Zn deficiency did not increase Cu concentration of the root under 5 μM Cu treatment (Fig. 3), but increased 1.8 times under 20 μM Cu treatment ($P<0.05$), suggesting that Zn deficiency could induce Cu absorption in root under high excess Cu.

Metal Accumulation in Root Affected by Cu

Interactions between metals depend on Cu exposure. Cu deficiency and Cu excess differently alter these interactions and the balance among specific micronutrients. The effect of Cu deficiency on Fe, Mn, and Zn uptake was shown in Fig. 4. Fe concentrations in the Cu-deficient, treated roots were increased 150% compared with the control roots, while Mn and Zn concentrations were not promoted significantly by Cu deficiency ($P<0.05$).

Cu concentrations of the root increased, while Fe, Mn, and Zn concentrations decreased with the Cu treatment from 0.5 to 200 μM (Fig. 5), indicating Cu, Fe, Mn, and Zn interactions in deposition by roots. Mn deposition in roots was more sensitive to Cu

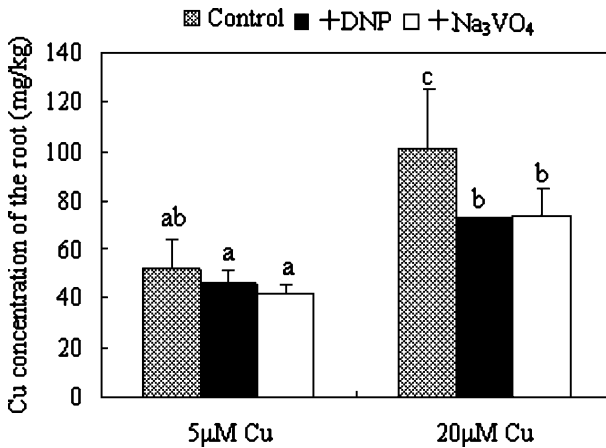


Fig. 1 Influence of the uncoupler and P-type ATPase inhibitor on the Cu uptake of *C. communis*. Seedlings were treated with 50 μM DNP or 50 μM Na₃VO₄ in nutrient solution containing 5 or 20 μM CuSO₄. Plants were harvested after 5 h. Values are means ± SD, $n=3$. Different letters (*a, b, c...*) in each histogram indicate a significant difference at the 0.05 level. The letters in this figure and those in Figs. 2, 3, 4 have the same meaning

treatment than Fe and Zn depositions. Fe and Zn depositions in roots were not affected by Cu significantly until the Cu-treated concentrations increased to 50 μM. However, Mn concentrations in roots decreased 1.3 times when Cu treatment concentrations increased from 0.5 to 20 μM.

Metal Distribution in Root Growth Zones

Metal (Cu, Fe, Zn, Mn, and Ca) distribution in root growth zones of *C. communis* is shown in Fig. 6. From the longitudinal section around the meristem, the high, relative Cu concentrations were observed in the meristem and epidermis, and Cu content was lower in the cortex than in the vascular cylinder (Fig. 6a). Similar to Cu, the high contents of Zn and

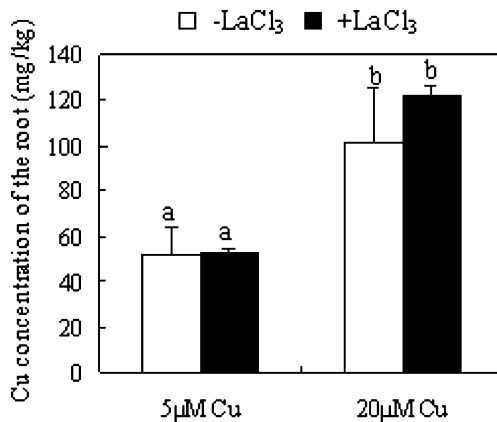


Fig. 2 Effect of the Ca²⁺ ion channel inhibitor on Cu uptake of *C. communis*. Seedlings were treated with 0.1 mM LaCl₃ for 48 h in the nutrient solutions containing 5 or 20 μM CuSO₄. Values are means ± SD, $n=3$

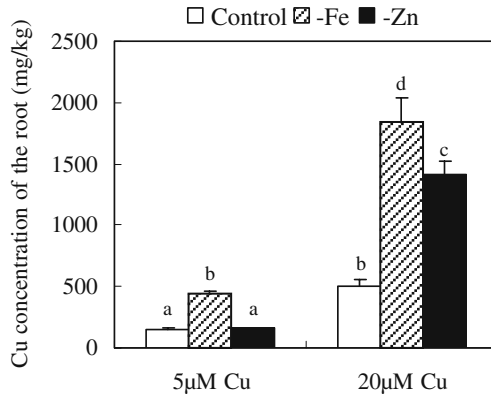


Fig. 3 Cu uptake in *C. communis* response to Fe or Zn deficiency. Seedlings were treated by Fe or Zn deficiency for 6 days, and then 5 or 20 μM CuSO₄ was used to study the Cu uptake under Fe or Zn deficiency. The seedlings were harvested after 48 h uptake. Values are means ± SD, *n*=3

Ca were observed in the meristem, while Fe and Mn contents in the meristem were not high.

In the cross section of elongation tissue, the Cu concentration decreased from the epidermis to the endodermis and reached the highest level in the vascular cylinder (Fig. 6b). In contrast, the highest Fe concentration was in the epidermis tissue and Fe concentration was higher in endodermis than in the vascular cylinder. Mn distribution and Zn distribution in the cross section of elongation tissue were more homogenous than that of Cu and Fe. Ca concentration was high in the epidermis and the vascular cylinder.

Compared with the elongation tissue, the obviously high concentrations of metals (including Cu, Fe, Mn, Zn, and Ca) were found in the epidermis of non-growing tissue (Fig. 6c). It was probably due to longer time of Cu treatment for non-growing tissue than that for elongation tissue. Metals were accumulated in the epidermis mostly by absorption and diffusion and then transported to cortex by the symplast way and the apoplast way. However, the symplast way was blocked in the endodermis because the cells were thickened like horse hoof, which made the solution be chosen into the vascular cylinder. The fact that Cu concentration was higher in the vascular cylinder than in the cortex

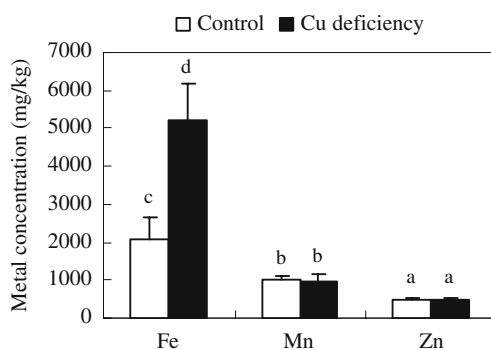


Fig. 4 Fe, Mn, and Zn uptake in *C. communis* response to Cu deficiency. Seedlings were treated by Cu deficiency for 6 days, and then the seedlings were harvested. Values are means ± SD, *n*=3

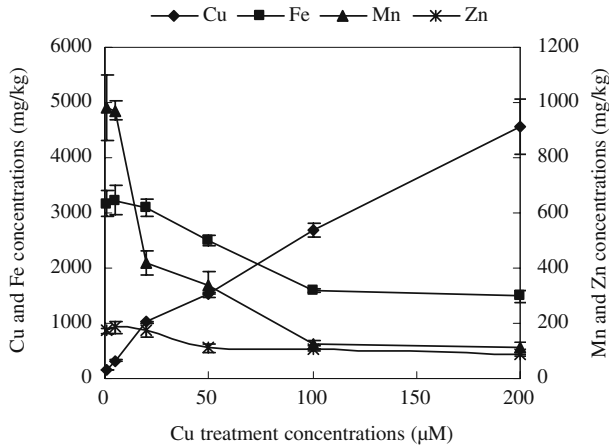


Fig. 5 Cu, Fe, Mn, and Zn concentrations in roots of *C. communis* under 0.5, 5, 20, 50, and 100 μM CuSO_4 treatment. Values are means \pm SD, $n=3$

demonstrated that the root of *C. communis* could uptake Cu actively. The data show that Cu interacts with different metals such as Fe, Mn, and Zn.

Discussion

Acquisition of metals by plants is a complex phenomenon [28–30]. The concentration of free metal in the cytoplasm is very small and is highly regulated. At the root level, the regulation includes ionic and covalent binding in cell walls, metal transport across the plasma membrane of root cells, xylem loading and translocation, and detoxification and sequestration of metal in cellular organelles or root hairs.

The Cu uptake by roots of *C. communis* was inhibited by the uncoupler DNP and P-type ATPase inhibitor Na_3VO_4 (Fig. 1), suggesting that Cu could probably be assimilated actively by roots and be related with P-type ATPase. P-type heavy metal ATPases have been identified in a wide range of organisms and have been implicated in the transport of a range of essential and also potentially toxic metals across cell membranes [31, 32]. There were five Cu-transporting P-type ATPases, as has been reported in *Arabidopsis*. PAA1, PAA2, and HMA1 are localized in the chloroplast and involved in the transport of Cu into the organelle, whereas RAN1 is involved in the Cu delivery to the secretory pathway and HMA5 would function in Cu compartmentalization or detoxification [30]. Ca-ATPases is a kind of P-type ATPase. Lanthanum is thought to block the dephosphorylation of the phosphoenzyme in the reaction cycle and result in an increase in its net equilibrium level, a reduction in turnover of the Ca^{2+} -ATPase and a reduction in calcium transport [26]. In this study, Cu uptake by the root was not inhibited by LaCl_3 (Fig. 2), suggesting that Cu uptake by *C. communis* was not through Ca^{2+} ion channel.

Metal interactions affecting distribution have been noted in various species of plants with additional metals having both positive and negative effects on uptake and deposition of a given metal. In this study, we found that Fe or Zn deficiency could enhance Cu uptake, while high levels of Cu inhibited Fe, Zn, and Mn accumulation in the root (Figs. 3 and 4), suggesting Cu interactions with Fe, Zn, and Mn. It was reported that Fe deficiency in the growth medium increased Cu concentration in maize and pea. Peralta-Videa et al. [33]

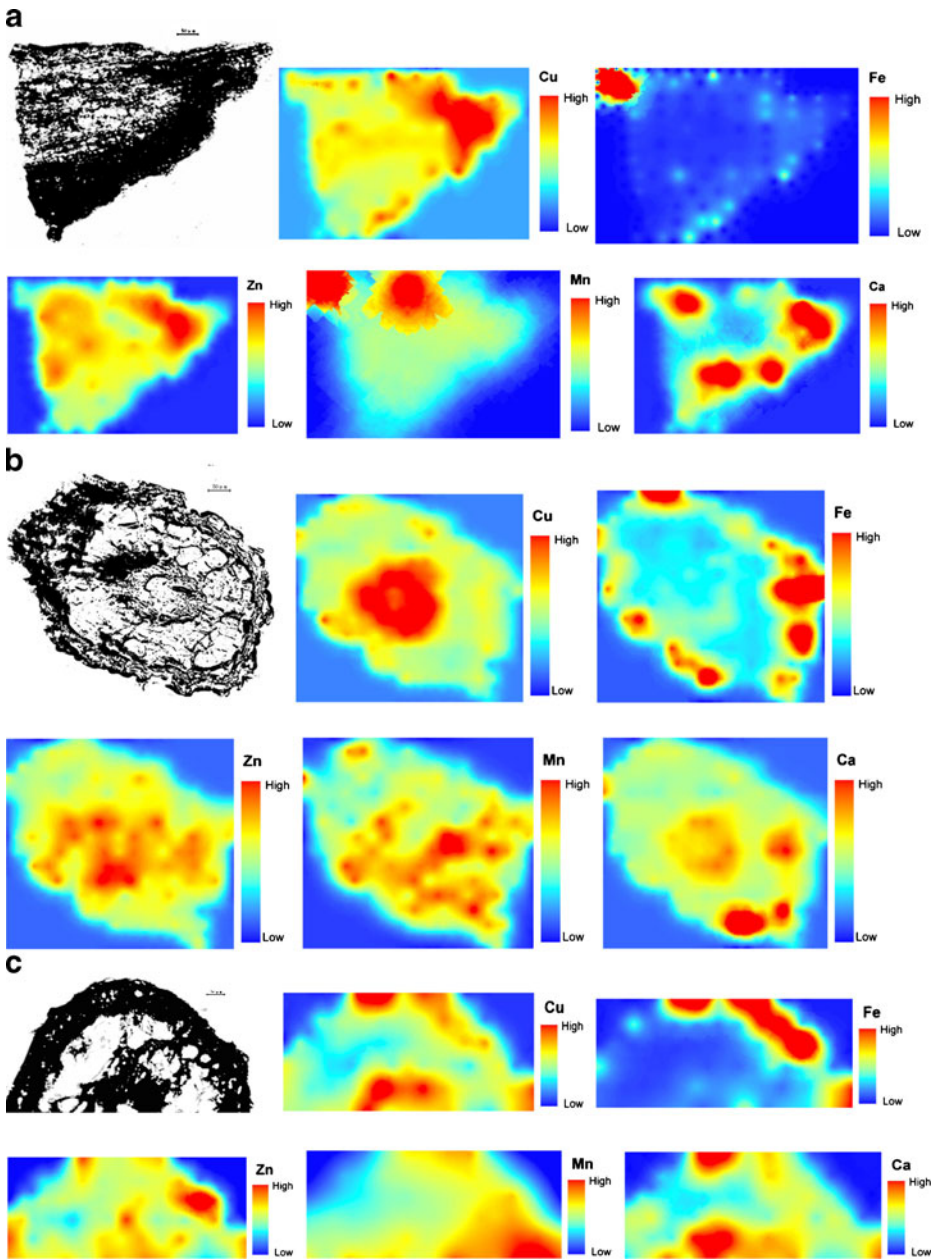


Fig. 6 Metal distribution of *C. communis* root response to 100 μM Cu revealed by SRXRF. **a** Longitudinal section around the meristem and metal locations, **b** Cross section of elongation tissue and metal locations, **c** Cross-section of non-growing tissue and metal locations. The scale bar of sections represents 50 μm , and the metals include Cu, Fe, Zn, Mn, and Ca

reported that the presence of Zn reduced Cu uptake in alfalfa plants (*Medicago sativa*). Chen et al. [34] reported that Fe deficiency induces Cu uptake and accumulation in *C. communis*. Bernal et al. [3] found that Cu accumulation was accompanied by Fe increase and Zn decrease in leaves treated with Cu, but the opposite was observed in leaves of plants treated by supplementing the growth medium with excess Cu. Metal interactions in roots have been reported to be mostly due to competition for the same membrane transporters [35]. The COPT (copper transporter) family plays a key role in Cu transport. COPT1 has been reported to be involved in Cu uptake [30]. There is no evidence that show whether COPT1 can mediate the transport of other metals. However, some studies suggest that IRT1 (iron-regulated transporter), a member of the ZIP (ZRT IRT-like protein) family, is able to mediate the transport of multiple metals, including Fe, Mn, Zn, Cd, and Cu [17, 36, 37].

SRXRF microprobe requires no sample pretreatment, which allows non-invasive examination of living materials, and can detect elemental abundances in the sub-microgram per gram range with a resolution of 10 μm or less, which was successfully completed in studies of metal localization in plants [23, 38]. The high levels of Cu were found in the meristem (Fig. 6a), indicating that vacuolar sequestration is not the primary detoxification mechanism in roots. The pattern is consistent with Cu being bound to the wall, since wall density is highest where cells are smallest, in the rapidly dividing zone, and crosswalls are progressively diluted by continuing cell expansion beyond the meristem. It was found that a large amount of Cu accumulated in *E. splendens* and was compartmentalized in the cell wall of roots [11, 39]. The higher Cu concentration in the vascular cylinder than in the endodermis (Fig. 6b, c) further proved the active Cu transport by roots. The Casparian strip and endodermis seem to provide selectivity against Cu, with reduced concentrations entering the stele [10]. Vesik [40] found an increasing Cu gradient from the epidermis through the root cortex of *Eichhornia crassipes*. However, Cu in the aquatic fern was found to decrease in the stele [41].

The results of metal localization in root growth zones revealed by SRXRF (Fig. 6) provided good information for us to further understand the metal interaction in roots, especially for Cu and Fe interaction. Under excess Cu stress, the highest levels of Fe were observed in epidermis tissue, and Fe levels were higher in the endodermis than in the vascular cylinder (Fig. 6), suggesting that Cu and Fe compete probably not only for the transporter (such as IRT1) in epidermal cells but also for the intercellular and intracellular transport in roots. Members of several different transport families have been implicated in the intracellular and intercellular metal transport including the Nramp (natural-resistance-associated macrophage protein) family and the YSL (yellow stripe-like) family [30].

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