1 **Title:**

2 Spatial imaging and speciation of Cu in rice (*Oryza sativa* L.) roots using synchrotron-based
3 X-ray microfluorescence and X-ray absorption spectroscopy

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23 **Abstract**

24 Knowledge of elemental localization and speciation in rice (*Oryza sativa* L.) roots is crucial
25 for elucidating the mechanisms of Cu accumulation so as to facilitate the development of
26 strategies to inhibit Cu accumulation in rice grain grown in contaminated soils. Using
27 synchrotron-based X-ray microfluorescence and X-ray absorption spectroscopy, we
28 investigated the distribution patterns and speciation of Cu in rice roots treated with 50 μ M Cu
29 for 7 days. A clear preferential localization of Cu in the meristematic zone was observed in
30 root tips as compared with the elongation zone. Investigation of Cu in the root cross sections
31 revealed that the intensity of Cu in the vascular bundles was more than 10-fold higher than
32 that in the other scanned sites (epidermis and cortex) in rice roots. The dominant chemical
33 form of Cu (79.1%) in rice roots was similar to that in the Cu-cell wall compounds. These
34 results suggest that although Cu can be easily transported into the vascular tissues in rice
35 roots, most of the metal absorbed by plants is retained in the roots owing to its high binding
36 to the cell wall compounds, thus preventing metal translocation to the aerial parts of the
37 plants.

38 **Keywords:** Rice; Copper; Root; Localization; Speciation

39 **Abbreviations** μ -XRF, micro X-ray fluorescence; XANES, X-ray absorption near edge
40 structure

41 **1. Introduction**

42 As a global staple food, rice (*Oryza sativa* L.) provides daily diet to millions of people
43 worldwide and also directly supplies about 19% of all calories consumed by the whole
44 population worldwide (Elert, 2014). On the other hand, rice is also one of the significant
45 dietary sources of heavy metals in foods (Who, 2011). Heavy metal contamination in
46 croplands can result in dietary exposure through the soil–plant–food chain transfer, which
47 causes elevated levels of toxic metals in human organs (Yang et al., 2016). In recent years,
48 copper (Cu) contamination is becoming a severe problem in many regions and environments.
49 Cu is extensively released through human activities, such as industrial processes, pesticide
50 application, and mining, and presents a major concern for sustainable agrifood production,
51 especially in developing countries (Sudo et al., 2008; Adrees et al., 2015). With the
52 continually growing demand of rice and widespread contamination of Cu in soils, it is
53 important to understand how rice plants take up, transport, and store the metal.

54 The amount of Cu in plants varies considerably within a narrow range and depends on both
55 the plant species and the Cu-feeding status (Burkhead et al., 2009). Whereas alfalfa and
56 barley are highly tolerant to Cu, rice is much less tolerant to Cu stress and is also more
57 susceptible to Cu toxicity than other heavy metals, such as Ni, Co, and Zn (Sudo et al., 2008).

58 As the first barrier of mineral elements absorption from soils, roots play a key role in the
59 uptake of heavy metal ions (DalCorso et al., 2013). Rice roots are known to be particularly
60 sensitive to Cu toxicity owing to their high accumulation of Cu (Li et al., 2009; Thounaojam

61 et al., 2012). On the other hand, the high accumulation of Cu in roots is one of the exclusion
62 mechanisms that prevent the subsequent transport of Cu to the shoots, thus reducing the metal
63 toxicity in rice (Thounaojam et al., 2012). Therefore, knowledge of the physiological bases
64 governing root retention of Cu is critical to develop strategies for increasing Cu tolerance and
65 reducing the metal accumulation in the edible portions of rice grown in Cu-contaminated
66 soils.

67 As the most critical part of the root, the root tip (the region at the apex housing the apical
68 meristem and root cap) plays a vital role in plant health and is also the site most sensitive to
69 heavy metals (Kahle, 1993; Hawes et al., 2000). The rice root tip can be subdivided into three
70 zones: apical meristems, elongation zone, and maturation zone (Coudert et al., 2010). The
71 characterization of Cu and other mineral elements allocation in different root zones in rice is
72 fundamental and crucial for understanding its elemental homeostasis and defensive strategies.
73 However, most studies on Cu compartmentation in rice roots have relied on bulk analysis
74 (Ando et al., 2013; Yu et al., 2014; Zhang et al., 2014). Although localization and speciation
75 of Cu has been reported in the roots of plants such as cowpea (Kopittke et al., 2011), little
76 information is available on the metal spatial distribution and speciation in roots of crop
77 species. In recent years, a dramatic improvement has been achieved in the utilities of
78 synchrotron-based techniques such as X-ray fluorescence (XRF) and X-ray absorption
79 spectroscopy (XAS) for the analysis of metal distribution and speciation in biological
80 samples (Punshon et al., 2009; Tian et al., 2009; Tian et al., 2011; Lu et al., 2013a; Lu et al.,

2013b; Taylor et al., 2013). In the present study, XRF and XAS analyses were used to investigate the in vivo characteristics of Cu distribution patterns and its speciation model in root tips of rice.

2. Materials and methods

2.1 Plant culture

The seeds of Japonica rice (*Oryza sativa* L.) Nipponbare were kindly supplied by the Zhejiang Academy of Agricultural Sciences. Surface-sterilized rice seeds were germinated at 25°C in the dark. After germination, the plantlets were sowed in a pot filled with sterilized quartz sand in a growth chamber. Uniform seedlings at the three-leaf stage were transplanted to a 2.5-L black plastic pot with nutrient solution containing (in mM) NH_4NO_3 , 1.43; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.00; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.64; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.32; and K_2SO_4 , 1.32 and (in μM) $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 5.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.075; H_3BO_3 , 1.90; Fe-EDTA, 20.0; and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 (Yang et al., 1994). The nutrient solutions were replaced every 3 days and adjusted to $\text{pH } 5.5 \pm 0.1$ every day with NaOH or HCl. Plants were grown in a growth chamber under a photo flux density of $400 \mu\text{mol}/\text{m}^2/\text{s}$, a light/dark period of 16/8 h, day/night temperatures of 30/25°C, and day/night relative humidity of 75/85%.

2.2 Plant treatment and sample preparation

After 2 weeks, seedlings were treated with 50 μM CuSO_4 in nutrient solution for 7 days. Each treatment was replicated four times. At harvest, seedling roots were immersed in 1.0

100 mM EDTA for 5 min to remove surface-absorbed ions. Fresh roots were then cut from plants
101 and rinsed with deionized water. Root tips and maturation zone of fresh roots were
102 immediately cut, frozen with liquid nitrogen, and then freeze-dried under -20°C for 3 days.
103 Root sections (40 µm thick) were cut with a cryotome (Leica CM1950, Nussloch, Germany)
104 at a temperature of -20°C according to the method described in our previous study (Tian et al.,
105 2010). Sections in good condition were selected and freeze-dried at -20°C for 3 days.

106 **2.3 µ-XRF analysis**

107 µ-XRF imaging of elements for rice root samples was performed on Stanford Synchrotron
108 Radiation Lightsource (SSRL) beamline (BL) 2-3 using a Si (220) double-crystal
109 monochromator with harmonic rejection achieved by detuning one monochromator crystal to
110 50% peak intensity, with the storage ring Stanford Positron Electron Accelerating Ring
111 containing 90–100 mA at 3.0 GeV. The brilliance beam in BL2-3 was 1×10^{12}
112 photons/s/mm²/mrad²/0.1% BW. The 2-µm microfocused beam was produced by a
113 Kirkpatrick-Baez mirror pair (Xradia Inc., Pleasanton, CA, USA) with the sample at 45°
114 angle to the incident X-ray beam. The fluorescence yield was detected using a single-channel
115 Vortex Si detector. µ-XRF maps were obtained for the following major and minor trace
116 elements: Cu, Fe, Mn, K, Ca, P, S, and Cl. The centroid of the Zn K_α fluorescence is 8630 eV,
117 which is not easily separated from the Cu K_β at 8904 eV due to the high intensity of Cu in the
118 plant samples. The elemental distribution maps were collected using a 10-µm pixel size for
119 the root tips (Figures 1, 2, and 3), a 5-µm pixel size for the root cross sections (Figures 5 and

6), and a 20- μm pixel size for the maps (Figure 4). Dwell time per point was 200 ms. The beam energy was set to 13.5 keV during mapping.

Fluorescence signal intensities for the elements analyzed by $\mu\text{-XRF}$ were translated to concentrations ($\mu\text{g cm}^{-2}$) in the cross sections of each plant sample for quantitative analysis.

Known XRF calibration standards mounted on a 6- μm thick Mylar film (Micromatter, Vancouver, Canada) were imaged under the same conditions as the samples in BL2-3 at SSRL. Element concentrations were calculated in SMAK software by using measurements of the standards to obtain counts per second per $\mu\text{g cm}^{-2}$ and then divided by the pixel size (<http://www-ssrl.slac.stanford.edu/~swebb/smak.htm>). The red color, depicting elemental

concentrations in Figures 1 and 5, was scaled to the maximum value ($\mu\text{g cm}^{-2}$) for each map.

The fluorescence data in Figures 2, 4, and 6 were presented as tricolor maps that allow for the spatial distribution of three elements to be shown. Pixel brightness was displayed in RGB with the brightest spots corresponding to the highest element concentrations ($\mu\text{g cm}^{-2}$).

2.4 XAS analysis

XAS data of powdered rice roots after 7 days of exposure of 50 μM Cu were collected at the SSRL essentially as described previously (Tian et al., 2010). Cu K-edge spectra were recorded on BL7-3 with an upstream Rh-coated collimating mirror, a Si(220) double-crystal monochromator, and a downstream focusing mirror. The incident X-ray intensity was monitored using a Kr-filled ionization chamber. The monochromator energy of each spectrum was calibrated using Cu metal foil between the second and third ionization

140 chambers; its absorption edge was calibrated to 8979 eV. Copper K_{α} fluorescence was
141 recorded using a 30 element germanium detector (Canberra Industries, Meriden, CT, USA)
142 equipped with Soller slits.

143 Fresh root tips were ground under liquid nitrogen and pressed into 2-mm path-length Lucite
144 sample holders with Kapton tape windows cooled in liquid nitrogen. To minimize breakdown
145 and mixing of cellular components within the plant material, we carefully kept the tissue
146 frozen at all times during measurement. During data collection, samples were maintained at
147 approximately 10 K in a liquid helium flow cryostat to minimize the loss of intensity in the
148 signal. Model complexes of copper were measured as standards of the most important
149 potential types of ligands in plants. Spectra of standard Cu species including Cu-histidine,
150 Cu-cysteine, Cu^{2+} ($CuSO_4$ solution), Cu-malate, Cu-citrate, Cu-oxalate, and Cu-cell wall
151 were collected. All reference solutions were prepared in 30% glycerol to prevent ice crystal
152 formation. The complexes of Cu-histidine, Cu-cysteine, Cu-malate, Cu-citrate, and
153 Cu-oxalate were generated by adding 5.0 mM each of histidine, cysteine, malic acid, citric
154 acid, and oxalic acid to an aqueous solution of 0.5 mM $Cu(NO_3)_2$, pH 6. Root cell wall
155 material was exposed to 0.5 mM $Cu(NO_3)_2$ in hydroponic solution (final pH=6) for 72 h,
156 rinsed in deionized water, and frozen in liquid nitrogen for storage. Spectra of all plant
157 samples and standard solution samples were recorded in fluorescence mode. Six scans were
158 collected and averaged for the sample to improve the signal-to-noise ratio. The spectrum data
159 of each sample were energy normalized using the reference energy of the Cu foils, replicate

spectra for each sample were then merged, and data were background and baseline corrected using the SixPack program. For the X-ray absorption near edge structure (XANES), linear combination fitting (LCF) was performed using SixPack, and a maximum of three standards were permitted for each fit for the LCF.

2.5 Statistical analysis of data

Data were statistically analyzed using SPSS (version 12.0). Figures were created using the software Origin 8.0.

3. Results

3.1 Distribution patterns of Cu and other elements in bulk root tips of rice

After being exposed to 50 μM Cu for 7 days, bulk root tips were collected and analyzed by $\mu\text{-XRF}$ imaging. As shown in Figure 1, the analyzed root tips consist of different parts including the root cap, meristematic zone, and elongation zone. The elemental images analyzed by $\mu\text{-XRF}$ showed that the highest concentrations of Cu were observed at the root apex, particularly at the meristematic zone, but not at the epidermis of the root cap. Lower concentrations of Cu were noted at the elongation zone of the roots, with the exception of preferential accumulation of Cu in the center of the roots (possibly the cylinder tissues) (Figure 1). The distribution styles of the other elements were more or less different from those of Cu. The highest concentrations of Ca, K, P, and S were found at one side of the root cap and meristematic zone. A preferential localization to the cylinder of the elongation zone

179 was noted for most of the elements, but not for Fe and Mn. In contrast, the distribution
180 pattern of Fe was very different from that of the other elements, which showed its highest
181 accumulation in the epidermis of the roots.

Figure 1 Micro-XRF mapping of Cu and other nutrients (Fe, Mn, K, Ca, P, S, and Cl) in the root tips of rice (*Oryza sativa* L.) after treatments of 50 μ M Cu for 7 days. Fluorescence intensities of elements were normalized and subsequently translated to concentrations (μ g cm^{-2}) by using measurements of the standards. Scale bar: 200 μ m. E.Z., elongation zone; M.Z. meristematic zone; R.C., root cap; XRF, X-ray fluorescence.

The merged XRF images of Cu, Fe, and Mn clearly confirmed that Cu (red color) was of the highest concentrations in the meristematic zone of rice roots, whereas Fe (green color) was more uniformly distributed through the root tips (Figure 2A). On the basis of the intensity of the Cu signal (Figure 2B), the concentrations of Cu in the meristematic zone (line 1) were approximately 6-fold higher than those in the elongation zone (line 3). The highest level of Cu was observed in the center of the meristematic zone, with a concentration of up to 60 μ g cm^{-2} . The intensity of Fe was not significantly different between the different zones of root tips, with a preferential localization of this element in the epidermis, but a much lower level in the center (possibly the cylinder) of the root (Figure 2B). Similar as those treated with high Cu level (50 μ M), Cu in the controls exposed to nutrient solution (0.2 μ M Cu) also showed a preferential localization to root tips and cylinder, although the metal intensity is of much lower levels (Fig. 3).

Figure 2 Merged micro-XRF image [A] and concentrations [B] of Cu (red), Fe (green), and Mn (blue) in the root tips of rice (*Oryza sativa* L.) treated with 50 μM Cu for 7 days. [A] Pixel brightness is displayed in RGB with the brightest spots corresponding to the highest concentrations for Cu (red), Fe (green), and Mn (blue). [B] Concentrations ($\mu\text{g cm}^{-2}$) of Cu, Fe, and Mn in the selected scanning sites marked in [A]. L.1–L.3 represent lines 1–3, respectively. E.Z., elongation zone; M.Z., meristematic zone; R.C., root cap; Ep., epidermis; XRF, X-ray fluorescence. Scale bar: 200 μm .

Figure 3 Micro-XRF image of Cu in the root tips of rice (*Oryza sativa* L.) grown in nutrient solution containing 0.2 μM Cu. [A] Microscope of selected root tip. E.Z., elongation zone; M.Z., meristematic zone; R.C., root cap; Ep., epidermis; Scale bar: 200 μm . [B] XRF image of Cu in the root tip, fluorescence intensities of elements were normalized and subsequently translated to concentrations ($\mu\text{g cm}^{-2}$) by using measurements of the standards. [C] Concentrations ($\mu\text{g cm}^{-2}$) of Cu in the selected scanning sites marked in [B].

A separate experiment was performed to investigate the distribution patterns of Cu and other nutrients in the maturation zone of rice roots consisting of the main root and root hairs (Figure 4). The results indicated that Cu was not only localized preferentially in the main root but also quite abundant in the root hairs at the maturation zone of rice roots. A clear evidence of transport of Cu from the root hairs to main root was noted in this area (white arrow in Figure 4). Localization in both the main root and root hairs was also noted for K, and Ca; the other elements, including Fe, Mn, P, and S, were mostly restricted to the root hairs.

Figure 4 Micro-XRF mapping of Cu and other nutrients (Fe, K, Ca, Mn, Cl, P, and S) in the maturation zone of roots collected from rice (*Oryza sativa* L.) after treatments of 50 μM Cu for 7 days. Fluorescence intensities of elements were normalized and subsequently translated to concentrations ($\mu\text{g cm}^{-2}$) by using measurements of the standards. Scale bar: 200 μm . XRF, X-ray fluorescence

3.2 In situ spatial imaging of Cu and other elements in root cross section

To investigate in more detail the localization of elements in rice roots, we performed $\mu\text{-XRF}$ imaging of root cross sections by using a smaller step size (5 μm). The elemental distribution

232 maps of Cu, Fe, K, Ca, Mn, Cl, P, and S in the scanned areas of different regions of rice roots
233 are presented in Figure 5, together with photographs taken using an optical microscope. Each
234 map indicates the relative distribution of a specific element, and the scales of elemental
235 intensities ($\mu\text{g cm}^{-2}$) vary for each map. The results showed that Cu was mainly distributed in
236 the peripheral region of the cylinder, which likely corresponds to the vascular systems, and
237 considerably less Cu was observed in the epidermal and cortex tissues. Intensity analysis of
238 scan lines through the root cross section (Figure 6C) demonstrated that Cu concentrations
239 reached their maximum level of $3.5 \mu\text{g cm}^{-2}$ in the vascular bundle, possibly in the xylem
240 tissue, which plays a vital role in nutrient translocation from root to shoot. This is highly
241 consistent with what was observed in the whole root tips, wherein the preferential localization
242 of Cu is in the center of the roots (Figures 1 and 2).

Figure 5 Micro-XRF mapping of Cu and other nutrients (Fe, Mn, K, P, S, Ca, Mn, and Cl) in the root cross section of rice (*Oryza sativa* L.) after treatments of 50 μM Cu for 7 days. Fluorescence intensities of elements were normalized and subsequently translated to concentrations ($\mu\text{g cm}^{-2}$) by using measurements of the standards. Scale bar: 100 μm . Ep., epidermis; XRF, X-ray fluorescence.

Figure 6 Comparative distribution patterns of Cu, and Fe in the root cross section of rice (*Oryza sativa* L.) treated with 50 μM Cu for 7 days. [A] Microscope image of root cross section, Scale bar: 50 μm . Ep., epidermis. [B] Micro-XRF mapping of Cu (red), and Fe (green). Fluorescence intensities of elements were normalized and subsequently translated to concentrations by using measurements of the standards. Pixel brightness is displayed in RGB with the brightest spots corresponding to the highest concentrations ($\mu\text{g cm}^{-2}$) for the element depicted. XRF, X-ray fluorescence. Intensity ($\mu\text{g cm}^{-2}$) of Cu, and Fe in the selected scanning sites marked as white line was shown. Ep., epidermis; V., vascular systems in the cylinder of the roots. [C] Relationships between the X-ray intensity ($\mu\text{g cm}^{-2}$) of Cu and Fe for the measured root cross section of rice.

High concentrations of elements in the cylinder regions were noted for the other nutrients, such as K, Ca, P, and S, with the exception of Fe and Mn. The root appeared to contain substantial quantities of K, P, Ca, and S but lower quantities of Fe, and Mn (Figure 5). The elements of major nutrients (K, P, Ca, and S), particularly Ca, were also present in some areas of the cortex tissues besides the cylinder regions (Figure 5). The μ -XRF map of Fe, however, revealed an almost exclusive localization of the metal in the epidermis of the roots

(Figures 5, 6B). The concentration of Fe in the epidermis is likely to be many hundreds of times higher than that in the remainder of the root cross sections (Figure 6B). The element of Mn was also highly concentrated in the epidermis of root surface, but the effect was less pronounced than that of Fe. Statistical analysis showed that there was no correlation between the XRF intensities of Cu and Fe in root cross section (Figure 6C).

3.3 Speciation of Cu measured by XAS

Speciation of Cu in rice roots was determined by XAS under very low temperature using hydrated plant samples. XANES spectra of the plant samples are shown in Figure 7. The Cu K-edge XANES spectra of the root samples were quantitatively analyzed by LCF to a library of Cu standard spectra. For the root spectrum, a single component was first tested, and an additional component was allowed if normalized sum square (NSS) was decreased by at least 10%. Using this procedure, the spectra were correctly simulated by three components. Satisfactory fits were defined by NSS increase within 5% of that for the best fit. The curves representing the best-fit linear combinations are plotted as dashed lines in Figure 7. The corresponding fitting parameters are reported in Table 1. Three types of components were identified: Cu-cell wall, Cu-histidine, and Cu-cysteine. This analysis revealed that the dominant (up to 79.1%) form of Cu species in rice roots treated with 50 μ M for 7 days was similar to the Cu-cell wall complex, with the remaining proportion of the metal in the roots composed mainly of Cu-histidine and Cu-cysteine (Table 1). This suggests that almost all the absorbed aqueous Cu is converted into an organic Cu compound in rice roots. However, it

286 should be noted here that the fitting procedure of XAS is not free of uncertainties and
 287 limitations and largely depend on the choices of the standards used (Williams et al., 2009),
 288 the speciation of Cu with other compounds in rice roots can not be completely excluded.

289
 290 **Figure 7** Cu K-edge XANES spectra recorded on roots and model compounds (solid lines)
 291 with best linear combination fits (dashed black line) of rice (*Oryza sativa* L.) treated with 50
 292 μ M Cu for 7 days. All samples were analyzed in the frozen hydrated state. XANES, X-ray
 293 absorption near edge structure.

294

Table 1 The fitting results of the Cu K-edge XANES spectra of samples with a linear combination using the measured data sets of representative model compounds.

Sample	Linear combination fits			Goodness of fit	
	Cu-cell wall (%)	Cu-histidine (%)	Cu-cysteine (%)	R factor	χ^2
Root	79.1	16.5	4.6	0.000213	0.000060

Fitting was performed within the X-ray absorption near edge structure (XANES) spectra from 8960 to 9040 eV using IFEFFIT.

4. Discussion

Different growth zones of plant roots have specific physiological functions and different strategies of nutrient uptake and accumulation (Song et al., 2013a). The present study in the root tips of rice supports the idea that the meristematic region of the root tip is the most active zone for mineral elements absorption (Walker et al., 2003), as the accumulation of all tested elements, with the exception of Fe, was the highest in the meristematic region (Figures 1 and 2). This preferential localization to the meristematic region close to the root cap is particularly significant in the case of Cu. A similar distribution pattern of Cu in root tips of cucumber (*Cucumis sativus*) was observed through an investigation using μ -XRF, which showed the highest content of Cu in the root cap and meristematic zone (Song et al., 2013b). It is well known that the Casparian strip and vascular bundles are not fully formed in the root apex region (Marschner and Marschner, 1995). The high concentration of Cu in the meristematic zone therefore probably resulted from the radial movement of Cu from the epidermis to the stele by the apoplastic pathway in this area, where the metal cannot be translocated to the shoots via the vascular tissues.

On the other hand, the preferential localization of Cu to the center of the elongation zone (Figures 1 and 2) and the main root at the maturation zone (Figure 4) implied that a considerable amount of Cu has entered into the root stele. This is confirmed by the detailed investigation of Cu in the root cross section (Figures 5 and 6), which shows a very Cu strong signal within the cylinder. The rice root stele consists of alternating xylem and phloem vessels (Coudert et al., 2010) and is the most important root fraction responsible for the transportation of mineral elements and for human nutrition, as it is critical to control element transport to the shoot (including rice grain) (Lux et al., 2011). The root stele is generally limited by the pericycle cell layer, where the Casparian strip prevents the radial movement of minerals in the apoplastic pathway. The high concentrations of Cu within the cylinder therefore suggest that the metal can be taken up by the root cells and can easily enter into the vascular systems through the symplastic pathway in rice roots.

The high distribution of Cu to the root vascular tissues is in contrast with the findings of the previous studies conducted on plants including cowpea, common reed, cowpea, grey mangrove, and willow, where Cu was predominantly contained within the rhizodermis, outer cortex, or cortical parenchyma, but to a considerably lower extent in the vascular bundle (MacFarlane and Burchett, 2000; Manceau et al., 2008; Kopittke et al., 2011; Zimmer et al., 2011). One of the possible reasons for the controversial results is the different plant species used and presented in the present and previous studies. It has been reported that the uptake and translocation mechanism of Cu differed in strategy I and II plants (Ryan et al., 2013) and

335 that dicots have a higher pectin concentration and overall binding site density than monocots
336 (Guigues et al., 2016), which may also affect the uptake and transport of Cu. Another
337 possibility is the different Cu supplying levels applied, considering that the previous studies
338 mentioned above all exposed plants to low Cu levels, whereas rice in our study was exposed
339 to a much higher Cu stress. The point of view is supported by the consistency of our results
340 with those observed in a Cu-tolerant plant (*Commelina communis*) supplied with a high level
341 of Cu (Shi et al., 2011). Although more investigations are required to understand whether the
342 transport and translocation mechanism could be concentration specific for Cu in rice roots,
343 the present study is, to our knowledge, the first to map the element distributions in rice root
344 under Cu stress in high resolution, which provides an in situ evidence for symplastic transport
345 of Cu in rice roots.

346 It has been suggested that the preferential accumulation of Cu in roots is probably owing to
347 the high affinity of Cu with hard ligands of the cell walls (Kopittke et al., 2014b). Although
348 the chemical speciation of Cu is still controversial, many studies have indicated that Cu was
349 typically sequestered in the cell walls to remove excess Cu in plants (Manceau et al., 2008;
350 Kopittke et al., 2011; Song et al., 2013b). In the present study, the investigation of Cu species
351 by using extended X-ray absorption fine structure (EXAFS) confirmed that the dominant
352 form of Cu species in rice roots was highly similar to the Cu-cell wall complex, which
353 suggested a possible role of the cell wall component involved in Cu speciation in rice roots.
354 The similar accumulation of Cu to root cell walls was also observed in rice plants exposed to

CuO nanoparticles by analysis of soft X-ray scanning transmission microscopy (Peng et al., 2015). Considering the high localization of Cu within the cylinders (Figures 1, 2, 5, and 6), we concluded that although Cu can be easily transported into the vascular tissues in rice roots, most of the Cu absorbed by plants is retained in the roots owing to its high binding to the cell wall compounds (and also the similar xylem tissues), thus preventing metal translocation to the aerial parts of the plants.

As compared with those of Cu, the characteristics of Fe and Mn distributed in rice roots are also worth mentioning. The epidermal localization of Fe (and Mn) is inconsistent with the findings of the previous studies showing that Fe and Mn were all accumulated at the root surface as redox-sensitive elements (Zimmer et al., 2011). It has been suggested that Fe can deposit as plaques around roots of plants including rice (Moore et al., 2011; Xu and Yu, 2013; Kopittke et al., 2014a), which is a general feature of rice roots growing in contaminated soils, providing a barrier for the potential absorption of some toxic elements (Hansel et al., 2002; Moore et al., 2011). Although Fe plaques cannot reduce Cu transport into the roots, it can enhance rice tolerance to high Cu, which probably resulted from the passivation of Cu by Fe (Huang et al., 2009). We hypothesize that the deposition of mixed Fe-Mn plaques around the rice root may also constitute for adapting in metal stress environment. Nevertheless, the mechanism of metal interaction in rice roots is still largely unknown. More information is necessary to further understand metal homeostasis in plants.

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References

- Adrees, M., Ali, S., Rizwan, M., Ibrahim, M., Abbas, F., Farid, M., Zia-ur-Rehman, M., Irshad, M.K., Bharwana, S.A., 2015. The effect of excess copper on growth and physiology of important food crops: a review. *Environ Sci Pollut R* 22, 8148-8162.
- Ando, Y., Nagata, S., Yanagisawa, S., Yoneyama, T., 2013. Copper in xylem and phloem saps from rice (*Oryza sativa*): the effect of moderate copper concentrations in the growth medium on the accumulation of five essential metals and a speciation analysis of copper-containing compounds. *Funct Plant Biol* 40, 89-100.
- Burkhead, J.L., Reynolds, K.A.G., Abdel-Ghany, S.E., Cohu, C.M., Pilon, M., 2009. Copper homeostasis. *New Phytol* 182, 799-816.
- Coudert, Y., Perin, C., Courtois, B., Khong, N.G., Gantet, P., 2010. Genetic control of root development in rice, the model cereal. *Trends Plant Sci.* 15, 219-226.

400 DalCorso, G., Manara, A., Furini, A., 2013. An overview of heavy metal challenge in plants:
 401 from roots to shoots. *Metallomics* 5, 1117-1132.
 402 Elert, E., 2014. A good grain. *Nature* 514, S50-S51.
 403 Guigues, S., Bravin, M.N., Garnier, C., Masion, A., Chevassus-Rosset, C., Cazevieuille, P.,
 404 Doelsch, E., 2016. Involvement of nitrogen functional groups in high-affinity copper binding
 405 in tomato and wheat root apoplasts: spectroscopic and thermodynamic evidence. *Metallomics*
 406 8, 366-376.
 407 Hansel, C.M., La Force, M.J., Fendorf, S., Sutton, S., 2002. Spatial and temporal association
 408 of As and Fe species on aquatic plant roots. *Environ Sci Technol* 36, 1988-1994.
 409 Hawes, M.C., Gunawardena, U., Miyasaka, S., Zhao, X.W., 2000. The role of root border
 410 cells in plant defense. *Trends Plant Sci.* 5, 128-133.
 411 Huang, Y., Hu, Y., Liu, Y., 2009. Combined toxicity of copper and cadmium to six rice
 412 genotypes (*Oryza sativa* L.). *J Environ Sci-China* 21, 647-653.
 413 Kahle, H., 1993. Response of roots of trees to heavy-metals. *Environ Exp Bot* 33, 99-119.
 414 Kopittke, P.M., de Jonge, M.D., Wang, P., McKenna, B.A., Lombi, E., Paterson, D.J.,
 415 Howard, D.L., James, S.A., Spiers, K.M., Ryan, C.G., Johnson, A.A.T., Menzies, N.W.,
 416 2014a. Laterally resolved speciation of arsenic in roots of wheat and rice using
 417 fluorescence-XANES imaging. *New Phytol* 201, 1251-1262.
 418 Kopittke, P.M., Menzies, N.W., de Jonge, M.D., McKenna, B.A., Donner, E., Webb, R.I.,
 419 Paterson, D.J., Howard, D.L., Ryan, C.G., Glover, C.J., Scheckel, K.G., Lombi, E., 2011. In
 420 situ distribution and speciation of toxic copper, nickel, and zinc in hydrated roots of cowpea.
 421 *Plant Physiol* 156, 663-673.
 422 Kopittke, P.M., Menzies, N.W., Wang, P., McKenna, B.A., Wehr, J.B., Lombi, E., Kinraide,
 423 T.B., Blamey, F.P.C., 2014b. The Rhizotoxicity of Metal Cations Is Related to Their Strength
 424 of Binding to Hard Ligands. *Environ Toxicol Chem* 33, 268-277.
 425 Li, F., Shi, J.Y., Shen, C.F., Chen, G.C., Hu, S.P., Chen, Y.X., 2009. Proteomic
 426 characterization of copper stress response in *Elsholtzia splendens* roots and leaves. *Plant Mol*
 427 *Biol* 71, 251-263.
 428 Lu, L.L., Tian, S.K., Liao, H.B., Zhang, J., Yang, X.E., Labavitch, J.M., Chen, W.R., 2013a.
 429 Analysis of Metal Element Distributions in Rice (*Oryza sativa* L.) Seeds and Relocation
 430 during Germination Based on X-Ray Fluorescence Imaging of Zn, Fe, K, Ca, and Mn. *Plos*
 431 *One* 8.
 432 Lu, L.L., Tian, S.K., Zhang, J., Yang, X.E., Labavitch, J.M., Webb, S.M., Latimer, M.,
 433 Brown, P.H., 2013b. Efficient xylem transport and phloem remobilization of Zn in the
 434 hyperaccumulator plant species *Sedum alfredii*. *New Phytol* 198, 721-731.
 435 Lux, A., Martinka, M., Vaculik, M., White, P.J., 2011. Root responses to cadmium in the
 436 rhizosphere: a review. *J Exp Bot* 62, 21-37.
 437 MacFarlane, G.R., Burchett, M.D., 2000. Cellular distribution of copper, lead and zinc in the
 438 grey mangrove, *Avicennia marina* (Forsk.) Vierh. *Aquatic Botany* 68, 45-59.

439 Manceau, A., Nagy, K.L., Marcus, M.A., Lanson, M., Geoffroy, N., Jacquet, T.,
440 Kirpichtchikova, T., 2008. Formation of metallic copper nanoparticles at the soil-root
441 interface. *Environ Sci Technol* 42, 1766-1772.

442 Marschner, H., Marschner, H., 1995. Mineral nutrition of higher plants, Second edition.

443 Moore, K.L., Schroeder, M., Wu, Z., Martin, B.G.H., Hawes, C.R., McGrath, S.P.,
444 Hawkesford, M.J., Ma, J.F., Zhao, F.J., Grovenor, C.R.M., 2011. High-resolution secondary
445 ion mass spectrometry reveals the contrasting subcellular distribution of arsenic and silicon in
446 rice roots. *Plant Physiol* 156, 913-924.

447 Peng, C., Duan, D., Xu, C., Chen, Y., Sun, L., Zhang, H., Yuan, X., Zheng, L., Yang, Y.,
448 Yang, J., Zhen, X., Chen, Y., Shi, J., 2015. Translocation and biotransformation of CuO
449 nanoparticles in rice (*Oryza sativa* L.) plants. *Environmental Pollution* 197, 99-107.

450 Punshon, T., Guerinot, M.L., Lanzirotti, A., 2009. Using synchrotron X-ray fluorescence
451 microprobes in the study of metal homeostasis in plants. *Ann. Bot* 103, 665-672.

452 Ryan, B.M., Kirby, J.K., Degryse, F., Harris, H., McLaughlin, M.J., Scheiderich, K., 2013.
453 Copper speciation and isotopic fractionation in plants: uptake and translocation mechanisms.
454 *New Phytol* 199, 367-378.

455 Shi, J., Yuan, X., Chen, X., Wu, B., Huang, Y., Chen, Y., 2011. Copper uptake and its effect
456 on metal distribution in root growth zones of *Commelina communis* revealed by SRXRF. *Biol*
457 *Trace Elem Res* 141, 294-304.

458 Song, J., Yang, Y.Q., Zhu, S.H., Chen, G.C., Yuan, X.F., Liu, T.T., Yu, X.H., Shi, J.Y.,
459 2013a. Spatial distribution and speciation of copper in root tips of cucumber revealed by
460 μ -XRF and μ -XANES. *Biol. Plant.* 57, 581-586.

461 Song, X.Q., Liu, L.F., Jiang, Y.J., Zhang, B.C., Gao, Y.P., Liu, X.L., Lin, Q.S., Ling, H.Q.,
462 Zhou, Y.H., 2013b. Disruption of secondary wall cellulose biosynthesis alters cadmium
463 translocation and tolerance in rice plants. *Mol Plant* 6, 768-780.

464 Sudo, E., Itouga, M., Yoshida-Hatanaka, K., Ono, Y., Sakakibara, H., 2008. Gene expression
465 and sensitivity in response to copper stress in rice leaves. *Journal of Experimental Botany* 59,
466 3465-3474.

467 Taylor, A., Day, M.P., Hill, S., Marshall, J., Patriarca, M., White, M., 2013. Atomic
468 spectrometry update. Clinical and biological materials, foods and beverages. *J Anal Atom*
469 *Spectrom* 28, 425-459.

470 Thounaojam, T.C., Panda, P., Mazumdar, P., Kumar, D., Sharma, G.D., Sahoo, L., Panda,
471 S.K., 2012. Excess copper induced oxidative stress and response of antioxidants in rice. *Plant*
472 *Physiol Bioch* 53, 33-39.

473 Tian, S.K., Lu, L.L., Labavitch, J., Yang, X.E., He, Z.L., Hu, H.N., Sarangi, R., Newville, M.,
474 Commisso, J., Brown, P., 2011. Cellular Sequestration of Cadmium in the Hyperaccumulator
475 Plant Species *Sedum alfredii*. *Plant Physiol* 157, 1914-1925.

476 Tian, S.K., Lu, L.L., Yang, X.E., Labavitch, J.M., Huang, Y., Brown, P., 2009. Stem and leaf
477 sequestration of zinc at the cellular level in the hyperaccumulator *Sedum alfredii*. *New Phytol*
478 182, 116-126.

Tian, S.K., Lu, L.L., Yang, X.E., Webb, S.M., Du, Y.H., Brown, P.H., 2010. Spatial Imaging and Speciation of Lead in the Accumulator Plant *Sedum alfredii* by Microscopically Focused Synchrotron X-ray Investigation. *Environ Sci Technol* 44, 5920-5926.

Walker, T.S., Bais, H.P., Grotewold, E., Vivanco, J.M., 2003. Root exudation and rhizosphere biology. *Plant Physiol* 132, 44-51.

Who, 2011. Evaluation of Certain Food Additives and Contaminants. Evaluation of Certain Food Additives and Contaminants. World health organization, distribution and sales service, 1211 27 Geneva, Switzerland.

Williams, P.N., Lombi, E., Sun, G.X., Scheckel, K., Zhu, Y.G., Feng, X.B., Zhu, J.M., Carey, A.M., Adomako, E., Lawgali, Y., Deacon, C., Meharg, A.A., 2009. Selenium Characterization in the Global Rice Supply Chain. *Environmental Science & Technology* 43, 6024-6030.

Xu, B., Yu, S., 2013. Root iron plaque formation and characteristics under N-2 flushing and its effects on translocation of Zn and Cd in paddy rice seedlings (*Oryza sativa*). *Ann. Bot* 111, 1189-1195.

Yang, J.L., Tang, C.L., Wang, F.W., Wu, Y.H., 2016. Co-contamination of Cu and Cd in paddy fields: Using periphyton to entrap heavy metals. *J Hazard Mater* 304, 150-158.

Yang, X., Romheld, V., Marschner, H., 1994. Effect of bicarbonate on root-growth and accumulation of organic-acids in Zn-efficient rice cultivars (*Oryza-sativa* L) *Plant Soil* 164, 1-7.

Yu, X.Z., Wang, D.Q., Zhang, X.H., 2014. Chelator-induced phytoextraction of zinc and copper by rice seedlings. *Ecotoxicology* 23, 749-756.

Zhang, L., Pan, Y.X., Lv, W., Xiong, Z.T., 2014. Physiological responses of biomass allocation, root architecture, and invertase activity to copper stress in young seedlings from two populations of *Kummerowia stipulacea* (maxim.) Makino. *Ecotox Environ Safe* 104, 278-284.

Zimmer, D., Kruse, J., Baum, C., Borca, C., Laue, M., Hause, G., Meissner, R., Leinweber, P., 2011. Spatial distribution of arsenic and heavy metals in willow roots from a contaminated floodplain soil measured by X-ray fluorescence spectroscopy. *Sci Total Environ* 409, 4094-4100.